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(71) Applicant: DIVERSA CORPORATION [US/US]; 10665 Sorrento Valley Road, San Diego, CA 92121 (US).

(72) Inventor: SHORT, Jay, M.; 320 Delage Drive, Encinitas, CA 92024 (US).

(74) Agent: HAILE, Lisa, A.; Fish & Richardson P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).

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(54) Title: SCREENING FOR NOVEL BIOACTIVITIES

(57) Abstract

Disclosed is a process for identifying clones having a specified activity of interest, which process comprises (i) generating one or more expression libraries derived from nucleic acid directly isolated from the environment; and (ii) screening said libraries utilizing an assay system. More particularly, this is a process for identifying clones having a specified activity of interest by (i) generating one or more expression libraries derived from nucleic acid directly or indirectly isolated from the environment; (ii) exposing said libraries to a particular substrate or substrates of interest; and (iii) screening said exposed libraries utilizing a fluorescence activated cell sorter to identify clones which react with the substrate or substrates. Also provided is a process for identifying clones having a specified activity of interest by (i) generating one or more expression libraries derived from nucleic acid directly or indirectly isolated from the environment; (ii) screening said exposed libraries utilizing an assay requiring a binding event or the covalent modification of a target, and a fluorescence activated cell sorter to identify positive clones.

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SCREENING FOR NOVEL BIOACTIVITIES

Field of the Invention

The present invention relates to the discovery of new bio-active molecules, such as antibiotics, anti-virals, anti-tumor agents and regulatory proteins. More particularly, the invention relates to a system for capturing genes potentially encoding novel biochemical pathways of interest in prokaryotic systems, and screening for these pathways utilizing high throughput screening assays.

Background of the Invention

Within the last decade there has been a dramatic increase in the need for bioactive compounds with novel activities. This demand has arisen largely from changes in worldwide demographics coupled with the clear and increasing trend in the number of pathogenic organisms that are resistant to currently available antibiotics. For example, while there has been a surge in demand for antibacterial drugs in emerging nations with young populations, countries with aging populations, such as the US, require a growing repertoire of drugs against cancer, diabetes, arthritis and other debilitating conditions. The death rate from infectious diseases has increased 58% between 1980 and 1992 and it has been estimated that the emergence of antibiotic resistant microbes has added in excess of $30 billion annually to the cost of health care in the US alone . (Adams *et al.*, *Chemical and Engineering News*, 1995; Amann *et al.*, *Microbiological Reviews*, 59, 1995). As a response to this trend pharmaceutical companies have significantly increased their screening of microbial diversity for compounds with unique activities or specificities.

There are several common sources of lead compounds (drug candidates), including natural product collections, synthetic chemical collections, and synthetic combinatorial chemical libraries, such as nucleotides, peptides, or other polymeric molecules. Each of these sources has advantages and disadvantages. The success of programs to screen these candidates depends largely on the number of compounds

entering the programs, and pharmaceutical companies have to date screened hundred of thousands of synthetic and natural compounds in search of lead compounds. Unfortunately, the ratio of novel to previously-discovered compounds has diminished with time. The discovery rate of novel lead compounds has not kept pace with demand despite the best efforts of pharmaceutical companies. There exists a strong need for accessing new sources of potential drug candidates.

The majority of bioactive compounds currently in use are derived from soil microorganisms. Many microbes inhabiting soils and other complex ecological communities produce a variety of compounds that increase their ability to survive and proliferate. These compounds are generally thought to be nonessential for growth of the organism and are synthesized with the aid of genes involved in intermediary metabolism hence their name - "secondary metabolites". Secondary metabolites that influence the growth or survival of other organisms are known as "bioactive" compounds and serve as key components of the chemical defense arsenal of both micro- and macroorganisms. Humans have exploited these compounds for use as antibiotics, antiinfectives and other bioactive compounds with activity against a broad range of prokaryotic and eukaryotic pathogens. Approximately 6,000 bioactive compounds of microbial origin have been characterized, with more than 60% produced by the gram positive soil bacteria of the genus *Streptomyces*. (Barnes *et al.*, *Proc.Nat. Acad. Sci. U.S.A.*, 91, 1994). Of these, at least 70 are currently used for biomedical and agricultural applications. The largest class of bioactive compounds, the polyketides, include a broad range of antibiotics, immunosuppressants and anticancer agents which together account for sales of over $5 billion per year.

Despite the seemingly large number of available bioactive compounds, it is clear that one of the greatest challenges facing modern biomedical science is the proliferation of antibiotic resistant pathogens. Because of their short generation time and ability to readily exchange genetic information, pathogenic microbes have rapidly evolved and disseminated resistance mechanisms against virtually all classes of antibiotic compounds. For example, there are virulent strains of the human pathogens *Staphylococcus* and *Streptococcus* that can now be treated with but a single antibiotic,

vancomycin, and resistance to this compound will require only the transfer of a single gene, *vanA*, from resistant *Enterococcus* species for this to occur . (Bateson *et al.*, *System. Appl. Microbiol*, 12, 1989). When this crucial need for novel antibacterial compounds is superimposed on the growing demand for enzyme inhibitors, immunosuppressants and anti-cancer agents it becomes readily apparent why pharmaceutical companies have stepped up their screening of microbial diversity for bioactive compounds with novel properties.

The approach currently used to screen microbes for new bioactive compounds has been largely unchanged since the inception of the field. New isolates of bacteria, particularly gram positive strains from soil environments, are collected and their metabolites tested for pharmacological activity. A more recent approach has been to use recombinant techniques to synthesize hybrid antibiotic pathways by combining gene subunits from previously characterized pathways. This approach, called "combinatorial biosynthesis" has focused primarily on the polyketide antibiotics and has resulted in a number of structurally unique compounds which have displayed activity. (Betz *et al.*, *Cytometry*, 5, 1984; Davey *et al.*, *Microbiological Reviews*, 60, 1989). However, compounds with novel antibiotic activities have not yet been reported; an observation that may be do to the fact that the pathway subunits are derived from those encoding previously characterized compounds. Dramatic success in using recombinant approaches due to small molecule synthesis has been recently reported in the engineering of biosynthetic pathways to increase the production of desirable antibiotics. (Diaper *et al.*, *Appl. Bacteriol.*, 77, 1994; Enzyme Nomenclature, *Academic Press*: NY, 1992).

There is still tremendous biodiversity that remains untapped as the source of lead compounds. However, the currently available methods for screening and producing lead compounds cannot be applied efficiently to these under-explored resources. For instance, it is estimated that at least 99% of marine bacteria species do not survive on laboratory media, and commercially available fermentation equipment is not optimal for use in the conditions under which these species will grow, hence these organisms are difficult or impossible to culture for screening or re-supply. Recollection, growth, strain improvement, media improvement and scale-up production of the drug-producing

organisms often pose problems for synthesis and development of lead compounds. Furthermore, the need for the interaction of specific organisms to synthesize some compounds makes their use in discovery extremely difficult. New methods to harness the genetic resources and chemical diversity of these untapped sources of compounds for use in drug discovery are very valuable. The present invention provides a path to access this untapped biodiversity and to rapidly screen for activities of interest utilizing recombinant DNA technology. This invention combines the benefits associated with the ability to rapidly screen natural compounds with the flexibility and reproducibility afforded with working with the genetic material of organisms.

Bacteria and many eukaryotes have a coordinated mechanism for regulating genes whose products are involved in related processes. The genes are clustered, in structures referred to as "gene clusters," on a single chromosome and are transcribed together under the control of a single regulatory sequence, including a single promoter which initiates transcription of the entire cluster. The gene cluster, the promoter, and additional sequences that function in regulation altogether are referred to as an "operon" and can include up to 20 or more genes, usually from 2 to 6 genes. Thus, a gene cluster is a group of adjacent genes that are either identical or related, usually as to their function. Gene clusters are of interest in drug discovery processes since product(s) of gene clusters include, for example, antibiotics, antivirals, antitumor agents and regulatory proteins.

Some gene families consist of one or more identical members. Clustering is a prerequisite for maintaining identity between genes, although clustered genes are not necessarily identical. Gene clusters range from extremes where a duplication is generated of adjacent related genes to cases where hundreds of identical genes lie in a tandem array. Sometimes no significance is discernable in a repetition of a particular gene. A principal example of this is the expressed duplicate insulin genes in some species, whereas a single insulin gene is adequate in other mammalian species.

Gene clusters undergo continual reorganization and, thus, the ability to create heterogeneous libraries of gene clusters from, for example, bacterial or other prokaryote sources is valuable in determining sources of novel bioactivities, including enzymes such

as, for example, the polyketide synthases that are responsible for the synthesis of polyketides having a vast array of useful activities.

Polyketides are molecules which are an extremely rich source of bioactivities, including antibiotics (such as tetracyclines and erythromycin), anti-cancer agents (daunomycin), immunosuppressants (FK506 and rapamycin), and veterinary products (monensin). Many polyketides (produced by polyketide synthases) are valuable as therapeutic agents. Polyketide synthases (PKSs) are multifunctional enzymes that catalyze the biosynthesis of a huge variety of carbon chains differing in length and patterns of functionality and cyclization. Despite their apparent structural diversity, they are synthesized by a common pathway in which units derived from acetate or propionate are condensed onto the growing chain in a process resembling fatty acid biosynthesis. The intermediates remain bound to the polyketide synthase during multiple cycles of chain extension and (to a variable extent) reduction of the (b-ketone group formed in each condensation. The structural variation between naturally occurring polyketides arises largely from the way in which each PKS controls the number and type of units added, and from the extent and stereochemistry of reduction at each cycle. Still greater diversity is produced by the action of regiospecific glycosylases, methyltransferases and oxidative enzymes on the product of the PKS.

Polyketide synthase genes fall into gene clusters. At least one type (designated type I) of polyketide synthases have large size genes and encoded enzymes, complicating genetic manipulation and *in vitro* studies of these genes/proteins. Progress in understanding the enzymology of such type I systems have previously been frustrated by the lack of cell-free systems to study polyketide chain synthesis by any of these multienzymes, although several partial reactions of certain pathways have been successfully assayed *in vitro*. Cell-free enzymatic synthesis of complex polyketides has proved unsuccessful, despite more than 30 years of intense efforts, presumably because of the difficulties in isolating fully active forms of these large, poorly expressed multifunctional proteins from naturally occurring producer organisms, and because of the relative lability of intermediates formed during the course of polyketide biosynthesis. In an attempt to overcome some of these limitations, modular PKS subunits have been

expressed in heterologous hosts such as *Escherichia coli* and *Streptomyces coelicolor*. Whereas the proteins expressed in *E. coli* are not fully active, heterologous expression of certain PKSs in *S.coelicolor* resulted in the production of active protein. Cell-free enzymatic synthesis of polyketides from PKSs with substantially fewer active sites, such as the 6-methylsalicylate synthase, chalcone synthase, tetracenomycin synthase, and the PKS responsible for the polyketide component of cyclosporin, have been reported.

Hence, studies have indicated that *in vitro* synthesis of polyketides is possible, however, synthesis was always performed with purified enzymes. Heterologous expression of genes encoding PKS modular subunits have allowed synthesis of functional polyketides *in vivo*, however, there are several challenges presented by this approach, which had to be overcome. The large sizes of modular PKS gene clusters (>30kb) make their manipulation on plasmids difficult. Modular PKSs also often utilize substrates which may be absent in a heterologous host. Finally, proper folding, assembly, and posttranslational modification of very large foreign polypeptides are not guaranteed.

Novel systems to clone and screen for bioactivities of interest *in vitro* are desirable. The method(s) of the present invention allow the cloning and discovery of novel bioactive molecules *in vitro*, and in particular novel bioactive molecules derived from uncultivated samples. Large size gene clusters can be cloned and screened using the method(s) of the present invention. Unlike previous strategies, the method(s) of the present invention allow one to clone utilizing well known genetic systems, and to screen *in vitro* with crude (impure) preparations.

Summary of the Invention

The present invention allows one to clone genes potentially encoding novel biochemical pathways of interest in prokaryotic systems, and screen for these pathways utilizing a novel process. Sources of the genes may be isolated, individual organisms ("isolates"), collections of organisms that have been grown in defined media ("enrichment cultures"), or, most preferably, uncultivated organisms ("environmental samples"). The use of a culture-independent approach to directly clone genes encoding

novel bioactivities from environmental samples is most preferable since it allows one to access untapped resources of biodiversity.

"Environmental libraries" are generated from environmental samples and represent the collective genomes of naturally occurring organisms archived in cloning vectors that can be propagated in suitable prokaryotic hosts. Because the cloned DNA is initially extracted directly from environmental samples, the libraries are not limited to the small fraction of prokaryotes that can be grown in pure culture. Additionally, a normalization of the environmental DNA present in these samples could allow more equal representation of the DNA from all of the species present in the original sample. This can dramatically increase the efficiency of finding interesting genes from minor constituents of the sample which may be under-represented by several orders of magnitude compared to the dominant species.

In the evaluation of complex environmental expression libraries, a rate limiting step occurs at the level of discovery of bioactivities. The present invention allows the rapid screening of complex environmental expression libraries, containing, for example, thousands of different organisms.

In the present invention, for example, gene libraries generated from one or more uncultivated microorganisms are screened for an activity of interest. Potential pathways encoding bioactive molecules of interest are first captured in prokaryotic cells in the form of gene expression libraries; crude or partially purified extracts, or pure proteins from metabolically rich cell lines are then combined with the gene expression libraries to create potentially active molecules; and the combination is screened for an activity of interest. Common approaches to drug discovery involve screening assays in which disease targets (macromolecules implicated in causing a disease) are exposed to potential drug candidates which are tested for therapeutic activity. In other approaches, whole cells or organisms that are representative of the causative agent of the disease, such as bacteria or tumor cell lines, are exposed to the potential candidates for screening purposes. Any of these approaches can be employed with the present invention.

The present invention also allows for the transfer of cloned pathways derived from uncultivated samples into metabolically rich hosts for heterologous expression and

downstream screening for bioactive compounds of interest using a variety of screening approaches briefly described above.

Accordingly, in one aspect, the present invention provides a process for identifying clones encoding a specified activity of interest, which process comprises (i) generating one or more expression libraries derived from nucleic acid directly isolated from the environment; and (ii) combining the expression libraries with crude or partially purified extracts, or pure proteins from metabolically rich cell lines; and (iii) screening said libraries utilizing any of a variety of screening assays to identify said clones.

In another aspect, the present invention provides a process for identifying clones encoding a specified activity of interest, which process comprises (i) generating one or more expression libraries derived from nucleic acid directly isolated from the environment; and (ii) transferring the clones into a metabolically rich cell line; and (iii) screening said cell line utilizing any of a variety of screening assays to identify said clones.

In another embodiment of the invention, expression libraries derived from DNA, primarily DNA directly isolated from the environment, are screened very rapidly for bioactivities of interest utilizing fluorescense activated cell sorting. These libraries can contain greater than 10^8 members and can represent single organisms or can represent the genomes of over 100 different microorganisms, species or subspecies.

Accordingly, in one aspect, the invention provides a process for identifying clones having a specified activity of interest, which process comprises (i) generating one or more expression libraries derived from nucleic acid directly isolated from the environment; and (ii) screening said libraries utilizing a high throughput cell analyzer, preferably a fluorescence activated cell sorter, to identify said clones.

More particularly, the invention provides a process for identifying clones having a specified activity of interest by (i) generating one or more expression libraries made to contain nucleic acid directly or indirectly isolated from the environment; (ii) exposing said libraries to a particular substrate or substrates of interest; and (iii) screening said exposed libraries utilizing a high throughput cell analyzer, preferably a

fluorescence activated cell sorter, to identify clones which react with the substrate or substrates.

In another aspect, the invention also provides a process for identifying clones having a specified activity of interest by (i) generating one or more expression libraries derived from nucleic acid directly or indirectly isolated from the environment; and (ii) screening said exposed libraries utilizing an assay requiring a binding event or the covalent modification of a target, and a high throughput cell analyzer, preferably a fluorescence activated cell sorter, to identify positive clones.

The invention further provides a method of screening for an agent that modulates the activity of a target protein or other cell component (*e.g.*, nucleic acid), wherein the target and a selectable marker are expressed by a recombinant cell, by co-encapsulating the agent in a micro-environment with the recombinant cell expressing the target and detectable marker and detecting the effect of the agent on the activity of the target cell component.

In another embodiment, the invention provides a method for enriching for target DNA sequences containing at least a partial coding region for at least one specified activity in a DNA sample by co-encapsulating a mixture of target DNA obtained from a mixture of organisms with a mixture of DNA probes including a detectable marker and at least a portion of a DNA sequence encoding at least one enzyme having a specified enzyme activity and a detectable marker; incubating the co-encapsulated mixture under such conditions and for such time as to allow hybridization of complementary sequences and screening for the target DNA. Optionally the method further comprises transforming host cells with recovered target DNA to produce an expression library of a plurality of clones. For example, transforming host cells iwth recovered gene librarires derived from the nucleic acid population to produce an expression library of a plurallity of clones.

The invention further provides a method of screening for an agent that modulates the interaction of a first test protein linked to a DNA binding moiety and a second test protein linked to a transcriptional activation moiety by co-encapsulating the agent with the first test protein and second test protein in a suitable microenvironment and determining the ability of the agent to modulate the interaction of the first test

protein linked to a DNA binding moiety with the second test protein covalently linked to a transcriptional activation moiety, wherein the agent enhances or inhibits the expression of a detectable protein. Preferably, screening is by FACS analysis.

In another embodiment the invention provides a means for selectively attracting microbes to specific substrates chemically conjugated to a solid surface. The invention further provides for the enrichment of these microbes. This approach allows for the concentration and collection of microbes, possessing genes encoding specific enzymes or small molecule pathways, from complex or dilute microbial populations in aqueous or terrestrial environments. The basis for the attraction and subsequent enrichment is that microbes possess specific receptors that signal chemotactic attraction towards specific substrates. By binding the substrate to a surface and subsequently incubating the substrate-surface conjugate in the presence of a mixed microbial population, specific members of that population can be collected.

It is a further object of the invention to provide a means for selectively enriching for specific microorganisms from the surrounding environmental matrix. In accomplishing these and other objects, there has been provided, in accordance with one aspect of the present invention, a device for collecting a population of microorganisms from an environmental sample comprising a solid support having a surface for attaching a selectable microbial enrichment media.

In one aspect of the invention, microbial enrichment media containing a microbial attractant is used to selectively lure members of the environmental community to the device. In another aspect of the invention, bioactive compounds which inhibit the growth of unwanted organisms is included in the microbial enrichment media to further enhance selection of desirable microorganisms.

In yet another aspect of the invention, a method for isolating microorganisms from an environmental sample comprising contacting the sample with a device having a solid support and a surface for attaching a selectable microbial enrichment media and isolating the population from the device is provided:

Brief Description of the Drawings

FIGURE 1 shows a scheme to capture, clone and archive large genome fragments from uncultivated microbes from natural environments. The cloning vectors used in this process can archive from 40 kbp (fosmids) to greater than 100 kbp (BACs).

FIGURE 2 shows the nucleotide alignment of a region of the ketosynthase I gene of polyketide pathways from a variety of *Streptomyces* species. These regions are aligned with a homologous region encoding a fatty acid synthase from *E. coli*. Observed sequence differences were used to construct probes that hybridize to cloned polyketide sequences but not to fatty acid sequences carried by the *E. coli* host strain.

FIGURE 3 shows an example of a high density filter array of environmental fosmid clones probed with a labeled oligonucleotide probe. The 2400 arrayed clones contain approximately 96 million base pairs of DNA cloned from a naturally occurring microbial community.

FIGURE 4 shows the results of mixed extract experiment measuring conferral of bioactivity on recombinant backbones heterologously expressed in *E. coli*.. **A**. Organic extracts from 3 oxytetracylin clones (1-3) and 3 gramicidin clones (4-6) were incubated with a protein extract from *Streptomyces lividans* strain TK24. After incubation the mixture was reextracted with methyl ethyl ketone, spotted on to filter disks, allowed to dry, then placed on a lawn of an *E. coli* test strain. Distinct zones of clearing can be seen around disks 2, 3 and 5. Extracts from 2 and 3 were subsequently seperated by thin layer chromatography which showed UV fluorescent spots with similar Rf and appearance to authentic oxytetracylin. **B**. Filters corresponding to those in **A** but without incubation with protein extract from *Streptomyces*. The *Streptomyces* extract alone also showed no bioactivity.

FIGURE 5 shows a strategy for FACS screening for recombinant bioactive molecules in *Streptomyces venezuelae*.

FIGURE 6 shows a micrograph of pMF4 oxytetracyclin clone expressed in *S. lividans* strain TK24. The red fluorescence near the end of the mycelia suggests that recombinant expression of oxytetracyclin may be induced at the onset sporulation as is the activity of the endogenous actinorhodin pathway.

FIGURE 7 shows an approach to screen for small molecules that enhance or inhibit transcription factor initiation. Both the small molecule pathway and the GFP reporter construct are co-expressed. Clones altered in GFP expression can then be sorted by FACS and the pathway clone isolated for characterization.

FIGURE 8 shows the gene replacement vector pLL25 designed to inactivate the actinorhodin pathway in *Streptomyces lividans* strain TK24.

FIGURE 9 shows the possible recombination events and predicted phenotypes from replacement of the actinorhodin gene cluster in *S. lividans* by the spectinomycin gene resident on pLL25.

FIGURE 10 shows a tandem duplication of a pMF3 clone into the S. lividans chromosome. Duplicated clones will contain *cos* sites at the appropriate spacing for lambda packaging.

Detailed Description of Preferred Embodiments

Sample Source/Collection

The method of the present invention begins with the construction of gene libraries which represent the collective genomes of naturally occurring organisms archived in cloning vectors that can be propagated in suitable prokaryotic hosts.

The microorganisms from which the libraries may be prepared include prokaryotic microorganisms, such as Eubacteria and Archaebacteria, and lower eukaryotic microorganisms such as fungi, some algae and protozoa. Libraries may be produced from environmental samples in which case DNA may be recovered without culturing of an organism or the DNA may be recovered from one or more cultured organisms. Such microorganisms may be extremophiles, such as hyperthermophiles, psychrophiles, psychrotrophs, halophiles, barophiles, acidophiles, *etc.*

The microorganisms from which the libraries may be prepared may be collected using a variety of techniques known in the art. Samples may also be collected using the methods detailed in the example provided below. Briefly, the example below provides a method of selective *in situ* enrichment of bacterial and archaeal species while at the same time inhibiting the proliferation of eukaryotic members of the population.

In situ enrichments can be performed to increase the likelihood of recovering rare species and previously uncultivated members of a microbial population. If one desires to obtain bacterial and archaeal species, nucleic acids from eukaryotes in an environmental sample can seriously complicate DNA library construction and decrease the number of desired bacterial species by grazing. The method described below employs selective agents, such as antifungal agents, to inhibit the growth of eukaryotic species.

In situ enrichment is achieved by using a microbial containment device composed of growth substrates and nutritional amendments with the intent to lure, selectively, members of the surrounding environmental matrix. Choice of substrates (carbon sources) and nutritional amendments (*i.e.*, nitrogen, phosphorous, etc.) is dependent upon the members of the community for which one desires to enrich. Selective agents against eukaryotic members are also added to the trap. Again, the exact composition depends upon which members of the community one desires to enrich and which members of the community one desires to inhibit. Some of the enrichment "media" which may be useful in pulling out particular members of the community is described in the example provided herein.

Sources of microorganism DNA as a starting material library from which target DNA is obtained are particularly contemplated to include environmental samples, such as microbial samples obtained from Arctic and Antarctic ice, water or permafrost sources, materials of volcanic origin, materials from soil or plant sources in tropical areas, etc. Thus, for example, genomic DNA may be recovered from either a culturable or non-culturable organism and employed to produce an appropriate recombinant expression library for subsequent determination of a biological activity. Prokaryotic expression libraries created from such starting material which includes DNA from more than one species are defined herein as multispecific libraries.

DNA Isolation

The preparation of DNA from the sample is an important step in the generation DNA libraries from environmental samples composed of uncultivated organisms, or for the generation of libraries from cultivated organisms. DNA can be

isolated from samples using various techniques well known in the art (Nucleic Acids in the Environment Methods & Applications, J.T. Trevors, D.D. van Elsas, Springer Laboratory, 1995). Preferably, DNA obtained will be of large size and free of enzyme inhibitors or other contaminants. DNA can be isolated directly from an environmental sample (direct lysis), or cells may be harvested from the sample prior to DNA recovery (cell separation). Direct lysis procedures have several advantages over protocols based on cell separation. The direct lysis technique provides more DNA with a generally higher representation of the microbial community, however, it is sometimes smaller in size and more likely to contain enzyme inhibitors than DNA recovered using the cell separation technique. Very useful direct lysis techniques have been described which provide DNA of high molecular weight and high purity (Barns, 1994; Holben, 1994). If inhibitors are present, there are several protocols which utilize cell isolation which can be employed (Holben, 1994). Additionally, a fractionation technique, such as the bis-benzimide separation (cesium chloride isolation) described herein, can be used to enhance the purity of the DNA.

Isolation of total genomic DNA from extreme environmental samples varies depending on the source and quantity of material. Uncontaminated, good quality (>20 kbp) DNA is required for the construction of a representative library for the present invention. A successful general DNA isolation protocol is the standard cetyl-trimethyl-ammonium-bromide (CTAB) precipitation technique. A biomass pellet is lysed and proteins digested by the nonspecific protease, proteinase K, in the presence of the detergent SDS. At elevated temperatures and high salt concentrations, CTAB forms insoluble complexes with denatured protein, polysaccharides and cell debris. Chloroform extractions are performed until the white interface containing the CTAB-complexes is reduced substantially. The nucleic acids in the supernatant are precipitated with isopropanol and resuspended in TE buffer.

For cells which are recalcitrant to lysis, a combination of chemical and mechanical methods with cocktails of various cell-lysing enzymes may be employed. Isolated nucleic acid may then further be purified using small cesium gradients.

A further example of an isolation strategy is detailed in an example below. This type of isolation strategy is optimal for obtaining good quality, large size DNA fragments for cloning.

Normalization

The present invention can further optimize methods for isolation of activities of interest from a variety of sources, including consortias of microorganisms, primary enrichments, and environmental "uncultivated" samples. Libraries which have been "normalized" in their representation of the genome populations in the original samples are possible with the present invention. These libraries can then be screened utilizing the methods of the present invention, for enzyme and other bioactivities of interest.

Libraries with equivalent representation of genomes from microbes that can differ vastly in abundance in natural populations are generated and screened. This "normalization" approach reduces the redundancy of clones from abundant species and increases the representation of clones from rare species. These normalized libraries allow for greater screening efficiency resulting in the identification of cells encoding novel biological catalysts.

In one embodiment, viable or non-viable cells isolated from the environment are, prior to the isolation of nucleic acid for generation of the expression gene library, FACS sorted to separate cells from the sample based on, for instance, DNA or AT/GC content of the cells. Various dyes or stains well known in the art, for example those described in "Practical Flow Cytometry", 1995 Wiley-Liss, Inc., Howard M. Shapiro, M.D., are used to intercalate or associate with nucleic acid of cells, and cells are separated on the FACS based on relative DNA content or AT/GC DNA content in the cells. Other criteria can be used to separate cells from the sample, as well. DNA is then isolated from the cells and used for the generation of expression gene libraries, which are then screened for activities of interest.

Alternatively, the nucleic acid is isolated directly from the environment and is, prior to generation of the gene library, sorted based on DNA or AT/GC content. DNA isolated directly from the environment, is used intact, randomly sheared or digested to

general fragmented DNA. The DNA is then bound to an intercalating agent as described above, and separated on the analyzer based on relative base content to isolate DNA of interest. Sorted DNA is then used for the generation of gene libraries, which are then screened for activities of interest.

As indicated, one embodiment for forming a normalized library from an environmental sample begins with the isolation of nucleic acid from the sample. This nucleic acid can then be fractionated prior to normalization to increase the chances of cloning DNA from minor species from the pool of organisms sampled. DNA can be fractionated using a density centrifugation technique, such as a cesium-chloride gradient. When an intercalating agent, such as bis-benzimide is employed to change the buoyant density of the nucleic acid, gradients will fractionate the DNA based on relative base content. Nucleic acid from multiple organisms can be separated in this manner, and this technique can be used to fractionate complex mixtures of genomes. This can be of particular value when working with complex environmental samples. Alternatively, the DNA does not have to be fractionated prior to normalization. Samples are recovered from the fractionated DNA, and the strands of nucleic acid are then melted and allowed to selectively reanneal under fixed conditions($C_o t$ driven hybridization). When a mixture of nucleic acid fragments is melted and allowed to reanneal under stringent conditions, the common sequences find their complementary strands faster than the rare sequences. After an optional single-stranded nucleic acid isolation step, single-stranded nucleic acid representing an enrichment of rare sequences is amplified using techniques well known in the art, such as a polymerase chain reaction (Barnes, 1994), and used to generate gene libraries. This procedure leads to the amplification of rare or low abundance nucleic acid molecules, which are then used to generate a gene library which can be screened for a desired bioactivity. While DNA will be recovered, the identification of the organism(s) originally containing the DNA may be lost. This method offers the ability to recover DNA from "unclonable" sources. This method is further detailed in the example below.

Hence, one embodiment for forming a normalized library from environmental sample(s) is by (a) isolating nucleic acid from the environmental sample(s); (b) optionally fractionating the nucleic acid and recovering desired fractions; (c) normalizing

the representation of the DNA within the population so as to form a normalized expression library from the DNA of the environmental sample(s). The normalization process is described and exemplified in detail in co-pending, commonly assigned U.S. Serial No. 08/665,565, filed June 18, 1996.

Gene Libraries

Gene libraries can be generated by inserting the normalized or non-normalized DNA isolated or derived from a sample into a vector or a plasmid. Such vectors or plasmids are preferably those containing expression regulatory sequences, including promoters, enhancers and the like. Such polynucleotides can be part of a vector and/or a composition and still be isolated, in that such vector or composition is not part of its natural environment. Particularly preferred phage or plasmids and methods for introduction and packaging into them are described herein.

The examples below detail procedures for producing libraries from both cultured and non-cultured organisms.

Cloning of DNA fragments prepared by random cleavage of the target DNA can also be used to generate a representative library. DNA dissolved in TE buffer is vigorously passed through a 25 gauge double-hubbed needle until the sheared fragments are in the desired size range. The DNA ends are "polished" or blunted with Mung Bean Nuclease, and EcoRI restriction sites in the target DNA are protected with EcoRI Methylase. EcoRI linkers (GGAATTCC) are ligated to the blunted/protected DNA using a very high molar ratio of linkers to target DNA. This lowers the probability of two DNA molecules ligating together to create a chimeric clone. The linkers are cut back with EcoRI restriction endonuclease and the DNA is size fractionated. The removal of sub-optimal DNA fragments and the small linkers is critical because ligation to the vector will result in recombinant molecules that are unpackageable, or the construction of a library containing only linkers as inserts. Sucrose gradient fractionation is used since it is extremely easy, rapid and reliable. Although the sucrose gradients do not provide the resolution of agarose gel isolations, they do produce DNA that is relatively free of

inhibiting contaminants. The prepared target DNA is ligated to the lambda vector, packaged using *in vitro* packaging extracts and grown on the appropriate *E. coli*.

As representative examples of expression vectors which may be used there may be mentioned viral particles, baculovirus, phage, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes, viral DNA (*e.g.* vaccinia, adenovirus, foul pox virus, pseudorabies and derivatives of SV40), P1-based artificial chromosomes, yeast plasmids, yeast artificial chromosomes, and any other vectors specific for specific hosts of interest (such as bacillus, aspergillus, yeast, *etc.*) Thus, for example, the DNA may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences. Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example; Bacterial: pQE vectors (Qiagen), pBluescript plasmids, pNH vectors, (lambda-ZAP vectors (Stratagene); ptrc99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene), pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia). However, any other plasmid or other vector may be used as long as they are replicable and viable in the host. Low copy number or high copy number vectors may be employed with the present invention.

A preferred type of vector for use in the present invention contains an f-factor origin replication. The f-factor (or fertility factor) in *E. coli* is a plasmid which effects high frequency transfer of itself during conjugation and less frequent transfer of the bacterial chromosome itself. A particularly preferred embodiment is to use cloning vectors, referred to as "fosmids" or bacterial artificial chromosome (BAC) vectors. These are derived from *E. coli* f-factor which is able to stably integrate large segments of genomic DNA. When integrated with DNA from a mixed uncultured environmental sample, this makes it possible to achieve large genomic fragments in the form of a stable "environmental DNA library."

Another preferred type of vector for use in the present invention is a cosmid vector. Cosmid vectors were originally designed to clone and propagate large segments of genomic DNA. Cloning into cosmid vectors is described in detail in Sambrook, et al.,

Molecular Cloning A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct RNA synthesis. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, *e.g.*, the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), a-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium.

The cloning strategy permits expression via both vector driven and endogenous promoters; vector promotion may be important with expression of genes whose endogenous promoter will not function in *E. coli*.

The DNA derived from a microorganism(s) may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA selected and isolated as hereinabove described is introduced into a suitable host to prepare a library which is screened for the desired activity. The selected DNA is preferably already in a vector which includes appropriate control sequences whereby selected DNA which encodes for a bio-activity may be expressed, for detection of the desired activity. The host cell is a prokaryotic cell, such as a bacterial cell. Particularly preferred host cells are *E.coli*. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)). The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Since it appears that many bioactive compounds of bacterial origin are encoded in contiguous multigene pathways varying from 15 to 100 kbp, cloning large genome fragments is preferred with the present invention, in order to express novel pathways from natural assemblages of microorganisms. Capturing and replicating DNA fragments of 40 to 100 kbp in surrogate hosts such as *E. coli* , *Bacillus* or *Streptomyces* is in effect "propagating" uncultivated microbes, albeit in the form of large DNA fragments each representing from 2 to 5% of a typical eubacterial genome.

Two hurdles that must be overcome to successfully capture large genome fragments from naturally occurring microbes and to express multigene pathways from subsequent clones are 1) the low cloning efficiency of environmental DNA and 2) the

inherent instability of large clones. To overcome these hurdles, high quality large molecular weight DNA is extracted directly from soil and other environments and vectors such as the F factor based Bacterial Artificial Chromosome (BAC) vectors are used to efficiently clone and propagate large genome fragments. The environmental library approach (Figure 1) will process such samples with an aim to archive and replicate with a high degree of fidelity the collective genomes in the mixed microbial assemblage. The basis of this approach is the application of modified Bacterial Artificial Chromosome (BAC) vectors to stably propagate 100-200 kbp genome fragments. The BAC vector and its derivative the fosmid (for F factor based cosmid) use the f-origin of replication to maintain copy number at one or two per cell. This feature has been shown to be a crucial factor in maintaining stability of large cloned fragments. High fidelity replication is especially important in propagating libraries comprised of high GC organisms such as the *Streptomyces* from which clones may be prone to rearrangement and deletion of duplicate sequences.

Because the fosmid vector uses the highly efficient lambda packaging system, comprehensive libraries can be assembled with a minimal amount of starting DNA. Environmental fosmid libraries of $4X10^7$ clones of the present invention can be generated, each containing approximately 40 kbp of cloned DNA, from 100 ng of purified DNA collected from samples, including, for example, from the microbial containment device described herein.

A potential problem with constructing libraries for the expression of bioactive compounds in *E. coli* is that this gram-negative bacterium may not have the appropriate genetic background to express the compounds in their active form. One aspect of the present invention allows the efficient cloning of fragments in *E. coli* and the subsequent transfer to a different suitable host for expression and screening. Shuttle vectors, which allow propagation in two different types of hosts, can be utilized in the present invention to clone and propagate in bacterial hosts, such as *E. coli*, and transfer to alternative hosts for expression of active molecules. Such alternative hosts may include but are not limited to, for example, *Streptomyces* or *Bacillus*, or other metabolically rich hosts such as *Cyanobacteria*, *Myxobacteria*, etc. *Streptomyces lividans*, for example, may be used

as the expression host for the cloned pathways. This strain is routinely used in the recombinant expression of heterologous antibiotic pathways because it recognized a large number of promoters and appears to lack a restriction system (Guseck, T.W. & Kinsella, J.E., (1992) *Crit. Rev. Microbiol.* 18, 247-260).

In the present invention, the example below describes a shuttle vector which can be utilized. The vector is an *E. coli- Streptomyces* shuttle vector. This system allows one to stably clone and express large inserts (40kbp genome fragments). Chromosomally integrated recombinants can be recovered as the original fosmid to facilitate sequence characterization and further manipulation of positive clones. Replicons which allow regulation of the clone copy number in hosts can be utilized. For instance, the SPC2 replicon, a 32kb fertility plasmid that is present at one copy per cell in *Streptomyces coelicolor*, can be utilized. This replicon can be "tuned" by truncation to replicate at various copy number in *Streptomyces* hosts. For instance, replicative versions of integrative shuttle vectors may be designed containing the full length and truncated SCP2 replicon which will regulate the clone copy number in the *Streptomyces* host from 1 to 10 copies per cell.

In order to ensure that the bioactivity of the clones containing the putative polyketide or other clustered genes is not due to the activation of any resident gene cluster, the resident gene sequences can be removed from the host strain by gene replacement or deletion. An example is presented below.

Biopanning

After the expression libraries have been generated one can include the additional step of "biopanning" such libraries prior to transfer to a second host for expression screening. The "biopanning" procedure refers to a process for identifying clones having a specified biological activity by screening for sequence homology in a library of clones prepared by *(i)* selectively isolating target DNA, from DNA derived from at least one microorganism, by use of at least one probe DNA comprising at least a portion of a DNA sequence encoding an biological having the specified biological

activity; and *(ii)* transforming a host with isolated target DNA to produce a library of clones which are then processed for screening for the specified biological activity.

The probe DNA used for selectively isolating the target DNA of interest from the DNA derived from at least one microorganism can be a full-length coding region sequence or a partial coding region sequence of DNA for an known bioactivity. The original DNA library can be preferably probed using mixtures of probes comprising at least a portion of the DNA sequence encoding a known bioactivity having a desired activity. These probes or probe libraries are preferably single-stranded and the microbial DNA which is probed has preferably been converted into single-stranded form. The probes that are particularly suitable are those derived from DNA encoding bioactivities having an activity similar or identical to the specified bioactivity which is to be screened.

The probe DNA should be at least about 10 bases and preferably at least 15 bases. In one embodiment, an entire coding region of one part of a pathway may be employed as a probe. Conditions for the hybridization in which target DNA is selectively isolated by the use of at least one DNA probe will be designed to provide a hybridization stringency of at least about 50% sequence identity, more particularly a stringency providing for a sequence identity of at least about 70%.

Hybridization techniques for probing a microbial DNA library to isolate target DNA of potential interest are well known in the art and any of those which are described in the literature are suitable for use herein, particularly those which use a solid phase-bound, directly or indirectly bound, probe DNA for ease in separation from the remainder of the DNA derived from the microorganisms.

Preferably the probe DNA is "labeled" with one partner of a specific binding pair (i.e. a ligand) and the other partner of the pair is bound to a solid matrix to provide ease of separation of target from its source. The ligand and specific binding partner can be selected from, in either orientation, the following: (1) an antigen or hapten and an antibody or specific binding fragment thereof; (2) biotin or iminobiotin and avidin or streptavidin; (3) a sugar and a lectin specific therefor; (4) an enzyme and an inhibitor therefor; (5) an apoenzyme and cofactor; (6) complementary homopolymeric oligonucleotides; and (7) a hormone and a receptor therefor. The solid phase is

preferably selected from: (1) a glass or polymeric surface; (2) a packed column of polymeric beads; and (3) magnetic or paramagnetic particles.

Further, it is optional but desirable to perform an amplification of the target DNA that has been isolated. In this embodiment the target DNA is separated from the probe DNA after isolation. It is then amplified before being used to transform hosts. Long PCR (Barnes, WM, Proc. Natl. Acad. Sci, USA, (1994) Mar 15) can be used to amplify large DNA fragments (e.g., 35kb). The double stranded DNA selected to include as at least a portion thereof a predetermined DNA sequence can be rendered single stranded, subjected to amplification and reannealed to provide amplified numbers of selected double stranded DNA. Numerous amplification methodologies are now well known in the art.

The selected DNA is then used for preparing a library for further processing and screening by transforming a suitable organism. Hosts, particularly those specifically identified herein as preferred, are transformed by artificial introduction of the vectors containing the target DNA by inoculation under conditions conducive for such transformation.

The resultant libraries of transformed clones are then processed for screening for clones which display an activity of interest. Clones can be shuttled in alternative hosts for expression of active compounds, or screened using methods described herein.

In vivo biopanning may be performed utilizing a FACS-based machine. Complex gene libraries are constructed with vectors which contain elements which stabilize transcribed RNA. For example, the inclusion of sequences which result in secondary structures such as hairpins which are designed to flank the transcribed regions of the RNA would serve to enhance their stability, thus increasing their half life within the cell. The probe molecules used in the biopanning process consist of oligonucleotides labeled with reporter molecules that only fluoresce upon binding of the probe to a target molecule. These probes are introduced into the recombinant cells from the library using one of several transformation methods. The probe molecules bind to the transcribed target mRNA resulting in DNA/RNA heteroduplex molecules. Binding of the probe to

a target will yield a fluorescent signal which is detected and sorted by the FACS machine during the screening process.

Having prepared a multiplicity of clones from DNA selectively isolated from an organism, such clones are screened for a specific activity and to identify the clones having the specified characteristics.

The screening for activity may be effected on individual expression clones or may be initially effected on a mixture of expression clones to ascertain whether or not the mixture has one or more specified activities. If the mixture has a specified activity, then the individual clones may be rescreened for such activity or for a more specific activity. Alternatively, encapsulation techniques such as gel microdroplets, may be employed to localize multiple clones in one location to be screened on a FACS machine for positive expressing clones within the group of clones which can then be broken out into individual clones to be screened again on a FACS machine to identify positive individual clones. Screening in this manner using a FACS machine is fully described in Patent Application Number 08/876,276 filed June 16, 1997. Thus, for example, if a clone mixture has a desirable activity, then the individual clones may be recovered and rescreened utilizing a FACS machine to determine which of such clones has the specified desirable activity.

As described with respect to one of the above aspects, the invention provides a process for activity screening of clones containing selected DNA derived from a microorganism which process comprises:

- screening a library for specified bioactivity, said library including a plurality of clones, said clones having been prepared by recovering from genomic DNA of a microorganism selected DNA, which DNA is selected by hybridization to at least one DNA sequence which is all or a portion of a DNA sequence encoding a bioactivity having a desirable activity; and
- transforming a host with the selected DNA to produce clones which are further processed and/or screened for the specified bioactivity.

In one embodiment, a DNA library derived from a microorganism is subjected to a selection procedure to select therefrom DNA which hybridizes to one or more probe DNA sequences which is all or a portion of a DNA sequence encoding an activity having a desirable activity by:

(a) rendering the double-stranded genomic DNA population into a single-stranded DNA population;

(b) contacting the single-stranded DNA population of (a) with the DNA probe bound to a ligand under conditions permissive of hybridization so as to produce a double-stranded complex of probe and members of the genomic DNA population which hybridize thereto;

(c) contacting the double-stranded complex of (b) with a solid phase specific binding partner for said ligand so as to produce a solid phase complex;

(d) separating the solid phase complex from the single-stranded DNA population of (b);

(e) releasing from the probe the members of the genomic population which had bound to the solid phase bound probe;

(f) forming double-stranded DNA from the members of the genomic population of (e);

(g) introducing the double-stranded DNA of (f) into a suitable host to form a library containing a plurality of clones containing the selected DNA; and

(h) screening the library for the desired activity.

In another aspect, the process includes a preselection to recover DNA including signal or secretion sequences. In this manner it is possible to select from the genomic DNA population or nucleic acid population by hybridization as hereinabove described only DNA which includes a signal or secretion sequence. The following paragraphs describe the protocol for this embodiment of the invention, the nature and function of secretion signal sequences in general and a specific exemplary application of such sequences to an assay or selection process.

A particularly preferred embodiment of this aspect further comprises, after (a) but before (b) above, the steps of:

(a *i*) contacting the single-stranded DNA population of (a) with a ligand-bound oligonucleotide probe that is complementary to a secretion signal sequence unique to a given class of proteins under conditions permissive of hybridization to form a double-stranded complex;

(a *ii*) contacting the double-stranded complex of (a *i*) with a solid phase specific binding partner for said ligand so as to produce a solid phase complex;

(a *iii*) separating the solid phase complex from the single-stranded DNA population of (a);

(a *iv*) releasing the members of the genomic population which had bound to said solid phase bound probe; and

(a *v*) separating the solid phase bound probe from the members of the genomic population which had bound thereto.

The DNA which has been selected and isolated to include a signal sequence is then subjected to the selection procedure hereinabove described to select and isolate therefrom DNA which binds to one or more probe DNA sequences derived from DNA encoding a bioactivity having a desirable bioactivity.

This procedure of "biopanning" is described and exemplified in U.S. Serial No. 08/692,002, filed August 2, 1996.

Further, it is possible to combine all the above embodiments such that a normalization step is performed prior to generation of the expression library, the expression library is then generated, the expression library so generated is then biopanned, and the biopanned expression library is then screened using a high throughput cell sorting and screening instrument. Thus there are a variety of options: *i.e.* (i) one can just generate the library and then screen it; (ii) normalize the target DNA, generate the expression library and screen it; (iii) normalize, generate the library, biopan and screen; or (iv) generate, biopan and screen the library.

Alternatively, the library may be screened for a more specialized enzyme activity. For example, instead of generically screening for hydrolase activity, the library

may be screened for a more specialized activity, *i.e.* the type of bond on which the hydrolase acts. Thus, for example, the library may be screened to ascertain those hydrolases which act on one or more specified chemical functionalities, such as: (a) amide (peptide bonds), *i.e.* proteases; (b) ester bonds, *i.e.* esterases and lipases; (c) acetals, *i.e.*, glycosidases *etc.*

The library may, for example, be screened for a specified enzyme activity. For example, the enzyme activity screened for may be one or more of the six IUB classes; oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. The recombinant enzymes which are determined to be positive for one or more of the IUB classes may then be rescreened for a more specific enzyme activity.

The present invention may be employed for example, to identify new enzymes having, for example, the following activities which may be employed for the following uses:

Lipase/Esterase. enantioselective hydrolysis of esters (lipids)/ thioesters, resolution of racemic mixtures, synthesis of optically active acids or alcohols from *meso*-diesters, selective syntheses, regiospecific hydrolysis of carbohydrate esters, selective hydrolysis of cyclic secondary alcohols, synthesis of optically active esters, lactones, acids, alcohols, transesterification of activated/nonactivated esters, interesterification, optically active lactones from hydroxyesters, egio- and enantioselective ring opening of anhydrides, detergents, fat/oil conversion and cheese ripening.

Protease. Ester/amide synthesis, peptide synthesis, resolution of racemic mixtures of amino acid esters, synthesis of non-natural amino acids and detergents/protein hydrolysis.

Glycosidase/Glycosyl transferase. Sugar/polymer synthesis, cleavage of glycosidic linkages to form mono, di-and oligosaccharides, synthesis of complex oligosaccharides, glycoside synthesis using UDP-galactosyl transferase, transglycosylation of disaccharides, glycosyl fluorides, aryl galactosides; glycosyl transfer in oligosaccharide synthesis, diastereoselective cleavage of a-glucosylsulfoxides, asymmetric glycosylations, food processing and paper processing.

Phosphatase/Kinase. Synthesis/hydrolysis of phosphate esters, regio- and enantioselective phosphorylation, introduction of phosphate esters, synthesize phospholipid precursors, controlled polynucleotide synthesis, activate biological molecule, selective phosphate bond formation without protecting groups.

Mono/Dioxygenase. Direct oxyfunctionalization of unactivated organic substrates, hydroxylation of alkane, aromatics, steroids, epoxidation of alkenes, enantioselective sulphoxidation, regio- and stereoselective Bayer-Villiger oxidations.

Haloperoxidase. Oxidative addition of halide ion to nucleophilic sites, addition of hypohalous acids to olefinic bonds, ring cleavage of cyclopropanes, activated aromatic substrates converted to *ortho* and *para* derivatives1.3 diketones converted to 2-halo-derivatives, heteroatom oxidation of sulfur and nitrogen containing substrates, oxidation of enol acetates, alkynes and activated aromatic rings

Lignin peroxidase/Diarylpropane peroxidase. Oxidative cleavage of C-C bonds, oxidation of benzylic alcohols to aldehydes, hydroxylation of benzylic carbons, phenol dimerization, hydroxylation of double bonds to form diols, cleavage of lignin aldehydes.

Epoxide hydrolase. Synthesis of enantiomerically pure bioactive compounds, regio- and enantioselective hydrolysis of epoxide, aromatic and olefinic epoxidation by monooxygenases to form epoxides, resolution of racemic epoxides, hydrolysis of steroid epoxides.

Nitrile hydratase/nitrilase. Hydrolysis of aliphatic nitriles to carboxamides, hydrolysis of aromatic, heterocyclic, unsaturated aliphatic nitriles to corresponding acids, hydrolysis of acrylonitrile, production of aromatic and carboxamides, carboxylic acids (nicotinamide, picolinamide, isonicotinamide), regioselective hydrolysis of acrylic dinitrile, amino acids from hydroxynitriles

Transaminase. Transfer of amino groups into oxo-acids.

Amidase/Acylase. Hydrolysis of amides, amidines, and other C-N bonds, non-natural amino acid resolution and synthesis.

The clones which are identified as having the specified activity may then be sequenced to identify the DNA sequence encoding a bioactivity having the specified activity. Thus, in accordance with the present invention it is possible to isolate and

identify: (i) DNA encoding a bioactivity having a specified activity, (ii) bioactivities having such activity (including the amino acid sequence thereof) and (iii) produce recombinant molecules having such activity.

Screening

The present invention offers the ability to screen for many types of bioactivities. For instance, the ability to select and combine desired components from a library of polyketides and postpolyketide biosynthesis genes for generation of novel polyketides for study is appealing. The method(s) of the present invention make it possible to and facilitate the cloning of novel polyketide synthases, and other relevant pathways or genes encoding commercially relevant secondary metabolites, since one can generate gene banks with clones containing large inserts (especially when using vectors which can accept large inserts, such as the f-factor based vectors), which facilitates cloning of gene clusters.

Preferably, the gene cluster or pathway DNA is ligated into a vector, particularly wherein a vector further comprises expression regulatory sequences which can control and regulate the production of a detectable protein or protein-related array activity from the ligated gene clusters. Use of vectors which have an exceptionally large capacity for exogenous DNA introduction are particularly appropriate for use with such gene clusters and are described by way of example herein to include the f-factor (or fertility factor) of *E. coli*. As previously indicated, this f-factor of *E. coli* is a plasmid which affect high-frequency transfer of itself during conjugation and is ideal to achieve and stably propagate large DNA fragments, such as gene clusters from mixed microbial samples. Other examples of vectors include cosmids, bacterial artificial chromosome vectors (BAC vectors), and P1 vectors. Lambda vectors can also accommodate relatively large DNA molecules, have high cloning and packaging efficiencies and are easy to handle and store compared to plasmid vectors. λ-ZAP vectors (Stratagene Cloning Systems, Inc.) have a convenient subcloning feature that allows clones in the vector to be excised with helper phage into the pBluescript phagemid, eliminating the time involved in subcloning. The cloning site in these vectors lies downstream of the *lac*

promoter. This feature allows expression of genes whose endogenous promoter does not function in *E. coli*.

Gene expression libraries of the present invention, capturing potential pathways encoding bioactive molecules of interest can first be induced in prokaryotic cells to express desirable precursers (*e.g.* backbone molecules which will be capable of being modified) which can then be screened in another host system which allows the expression of active molecules. Particulary preferred prokaryotic cells are *E.coli* cells. Alternatively, crude or partially purified extracts, or pure proteins from metabolically rich cell lines can be combined with the original gene expression libraries to create potentially active molecules, which can then be screened for an activity of interest.

For example, gene libraries can be generated in *E.coli* as a host, and a shuttle vector as the vector, according to the examples provided herein. These libraries may then be screened using "hybridization screening". "Hybridization screening" is an approach used to detect pathways encoding compounds related to previously characterized small molecules which relies on the hybridization of probes to conserved genes within the pathway. This approach appears effective for the polyketide class of molecules which have highly conserved regions within the polyketide synthase genes in the pathway. Because of the highly conserved nature of these genes, hybridization of probes to high density filter arrays of clones from low complexity libraries is an effective approach to identify clones carrying potential full length pathways. Alternatively, multiplex PCR using primers designed against the conserved pathway genes can be used on DNA pools from clones arrayed in microtiter dish format.

Libraries made from complex communities require an enrichment procedure to increase the likelihood of identifying by hybridization any clones carrying homologous sequences. For example, the ~100 million base pairs of DNA immobilized on the filter shown in Fig. 3 represents approximately 5-fold coverage of 3 typical *Streptomyces* genomes. However, a gram of soil can contain approximately 10^6 bacterial cells representing over 10^4 species. Screening a library made from such a sample would require over 3,000 filters.

In such nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (*e.g.*, GC v. AT content), and nucleic acid type (*e.g.*, RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter. An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1 x SSC at about 68°C (high stringency conditions). Washing can be carried out using only one of these conditions, *e.g.*, high stringency conditions, or each of the conditions can be used, *e.g.*, for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

The biopanning approach described above can be used to create libraries enriched with clones carrying sequences homologous to a given probe sequence. Using this approach libraries containing clones with inserts of up to 40 kbp can be enriched approximately 1,000 fold after each round of panning. This enables one to reduce the above 3,000 filter fosmid library to 3 filters after 1 round of biopanning enrichment. This approach can be applied to create libraries enriched for clones carrying polyketide sequences.

Hybridization screening using high density filters or biopanning has proven an efficient approach to detect homologues of pathways containing conserved genes. To discover novel bioactive molecules that may have no known counterparts, however, other approaches are necessary. Another approach of the present invention is to screen in *E. coli* for the expression of small molecule ring structures or "backbones". Because the genes encoding these polycyclic structures can often be expressed in *E. coli* the small molecule backbone can be manufactured albeit in an inactive form. Bioactivity is conferred upon transferring the molecule or pathway to an appropriate host that expresses

the requisite glycosylation and methylation genes that can modify or "decorate" the structure to its active form. Thus, inactive ring compounds, recombinantly expressed in *E. coli* are detected to identify clones which are then shuttled to a metabolically rich host, such as *Streptomyces*, for subsequent production of the bioactive molecule. The use of high throughput robotic systems allows the screening of hundreds of thousands of clones in multiplexed arrays in microtiter dishes.

One approach to detect and enrich for clones carrying these structures is to use FACS screening, a procedure described and exemplified in U.S. Serial No. 08/876,276, filed June 16, 1997. Polycyclic ring compounds typically have characteristic fluorescent spectra when excited by ultraviolet light. Thus clones expressing these structures can be distinguished from background using a sufficiently sensitive detection method. High throughput FACS screening can be utilized to screen for small molecule backbones in *E. coli* libraries. Commercially available FACS machines are capable of screening up to 100,000 clones per second for UV active molecules. These clones can be sorted for further FACS screening or the resident plasmids can be extracted and shuttled to *Streptomyces* for activity screening.

In an alternate screening approach, after shuttling to *Streptomyces* hosts, organic extracts from candidate clones can be tested for bioactivity by susceptibility screening against test organisms such as *Staphylococcus aureus*, *E. coli*, or *Saccharomyces cervisiae*. FACS screening can be used in this approach by co-encapsulating clones with the test organism (Fig. 5).

An alternative to the abovementioned screening methods provided by the present invention is an approach termed "mixed extract" screening. The "mixed extract" screening approach takes advantage of the fact that the accessory genes needed to confer activity upon the polycyclic backbones are expressed in metabolically rich hosts, such as *Streptomyces*, and that the enzymes can be extracted and combined with the backbones extracted from *E. coli* clones to produce the bioactive compound *in vitro*. Enzyme extract preparations from metabolically rich hosts, such as *Streptomyces* strains, at various growth stages are combined with pools of organic extracts from *E. coli*

libraries and then evaluated for bioactivity. A description of this is provided in the examples below.

Another approach to detect activity in the *E. coli* clones is to screen for genes that can convert bioactive compounds to different forms. For example, a recombinant enzyme was recently discovered that can convert the low value daunomycin to the higher value doxorubicin. Similar enzyme pathways are being sought to convert penicillins to cephalosporins.

In comparison to colorimetric assays, fluorescent based assays are very sensitive, which is a major criteria for single cell assays. There are two main factors which govern the screening of a recombinant enzyme in a single cell: i) the level of gene expression, and ii) enzyme assay sensitivity. To estimate the level of gene expression one can determine how many copies of the gene product will be produced by the host cell given the vector. For instance, one can assume that each *E. coli* cell infected with pBluescript phagemid (Stratagene Cloning Systems, Inc.) will produce ~10^3 copies of the gene product from the insert. The FACS instruments are capable of detecting about 500 to 1,000 fluorescein molecules per cell. Assuming that one enzyme turns over at least one fluorescein based substrate molecule, one cell will display enough fluorescence to be detected by the optics of a fluorescence-activated cell sorter (FACS).

Substrate can be administered to the cells before or during the process of the cell sorting analysis. In either case a solution of the substrate is made up and the cells are contacted therewith. When done prior to the cell sorting analysis this can be by making a solution which can be administered to the cells while in culture plates or other containers. The concentration ranges for substrate solutions will vary according to the substrate utilized. Commercially available substrates will generally contain instructions on concentration ranges to be utilized for, for instance, cell staining purposes. These ranges may be employed in the determination of an optimal concentration or concentration range to be utilized in the present invention. The substrate solution is maintained in contact with the cells for a period of time and at an appropriate temperature necessary for the substrate to permeablize the cell membrane. Again, this will vary with substrate. Instruments which deliver reagents in stream such as by poppet valves which

seal openings in the flow path until activated to permit introduction of reagents (*e.g.* substrate) into the flow path in which the cells are moving through the analyzer can be employed for substrate delivery.

The substrate is one which is able to enter the cell and maintain its presence within the cell for a period sufficient for analysis to occur. It has generally been observed that introduction of the substrate into the cell across the cell membrane occurs without difficulty. It is also preferable that once the substrate is in the cell it not "leak" back out before reacting with the biomolecule being sought to an extent sufficient to product a detectable response. Retention of the substrate in the cell can be enhanced by a variety of techniques. In one, the substrate compound is structurally modified by addition of a hydrophobic tail. In another certain preferred solvents, such as DMSO or glycerol, can be administered to coat the exterior of the cell. Also the substrate can be administered to the cells at reduced temperature which has been observed to retard leakage of the substrate from the cell's interior.

A broad spectrum of substrates can be used which are chosen based on the type of bioactivity sought. In addition where the bioactivity being sought is in the same class as that of other biomolecules for which a number have known substrates, the bioactivity can be examined using a cocktail of the known substrates for the related biomolecules which are already known. For example, substrates are known for approximately 20 commercially available esterases and the combination of these known substrates can provide detectable, if not optimal, signal production. Substrates are also known and available for glycosidases, proteases, phosphatases, and monoxygenases.

The substrate interacts with the target biomolecule so as to produce a detectable response. Such responses can include chromogenic or fluorogenic responses and the like. The detectable species can be one which results from cleavage of the substrate or a secondary molecule which is so affected by the cleavage or other substrate/ biomolecule interaction to undergo a detectable change. Innumerable examples of detectable assay formats are known from the diagnostic arts which use immunoassay, chromogenic assay, and labeled probe methodologies.

FACS screening can also be used to detect expression of UV fluorescent

molecules in metabolically rich hosts, such as *Streptomyces*. Recombinant oxytetracylin retains its diagnostic red fluorescence when produced heterologously in *S. lividans* TK24 (Fig. 6). Pathway clones, which can be sorted by FACS, can thus be screened for polycyclic molecules in a high throughput fashion.

Several enzyme assays described in the literature are built around the change in fluorescence which results when the phenolic hydroxyl (or anilino amine) becomes deacylated (or dealkylated) by the action of the enzyme. Figure 7 shows the basic principle for this type of enzyme assay for deacylation. Any emission or activation of fluorescent wavelengths as a result of any biological process are defined herein as bioactive fluoresence.

A variety of types of high throughput cell sorting instruments can be used with the present invention. First there is the FACS cell sorting instrument which has the advantage of a very high throughput and individual cell analysis. Other types of instruments which can be used are robotics instruments and time-resolved fluorescence instruments, which can actually measure the fluorescence from a single molecule over an elapsed period of time. Since they are measuring a single molecule, they can simultaneously determine its molecular weight, however their throughput is not as high as the FACS cell sorting instruments.

When screening with the FACS instrument, the trigger parameter is set with logarithmic forward side scatter. The fluorescent signals of positive clones emitted by fluorescein or other fluorescent substrates is distinguished by means of a dichroic mirror and acquired in log mode. For example, "active" clones can be sorted and deposited into microtiter plates. When sorting clones from libraries constructed from single organisms or from small microbial consortia, approximately 50 clones can be sorted into individual microtiter plate wells. When complex environmental mega-libaries (*i.e.* libraries containing ~10^8 clones which represent >100 organisms) about 500 expressing clones should be collected.

Plasmid DNA can then be isolated from the sorted clones using any commercially available automated miniprep machine, such as that from Autogen. The plasmids are then retransformed into suitable expression hosts and assayed for activity

utilizing chromogenic agar plate based or automated liquid format assays. Confirmed expression clones can then undergo RFLP analysis to determine unique clones prior to sequencing. The inserts which contain the unique esterase clones can be sequenced, open reading frames (ORF's) identified and the genes PCR subcloned for overexpression. Alternatively, expressing clones can be "bulk sorted" into single tubes and the plasmid inserts recovered as amplified products, which are then subcloned and transformed into suitable vector-hosts systems for rescreening.

Encapsulation techniques may be employed to localize signal, even in cases where cells are no longer viable. Gel microdrops (GMDs) are small (25 to 50um in diameter) particles made with a biocompatible matrix. In cases of viable cells, these microdrops serve as miniaturized petri dishes because cell progeny are retained next to each other, allowing isolation of cells based on clonal growth. The basic method has a significant degree of automation and high throughput; after the colony size signal boundaries are established, about 10^6 GMDs per hour can be automatically processed. Cells are encapsulated together with substrates and particles containing a positive clones are sorted. Fluorescent substrate labeled glass beads can also be loaded inside the GMDs. In cases of non-viable cells, GMDs can be employed to ensure localization of signal.

After viable or non-viable cells, each containing a different expression clone from the gene library are screened on a FACS machine, and positive clones are recovered, DNA is isolated from positive clones. The DNA can then be amplified either *in vivo* or *in vitro* by utilizing any of the various amplification techniques known in the art. *In vivo* amplification would include transformation of the clone(s) or subclone(s) of the clones into a viable host, followed by growth of the host. *In vitro* amplification can be performed using techniques such as the polymerase chain reaction.

All of the references mentioned above are hereby incorporated by reference in their entirety. Each of these techniques is described in detail in the references mentioned.

DNA can be mutagenized, or "evolved", utilizing any one or more of these techniques, and rescreened on the FACS machine to identify more desirable clones.

"Fluorescence screening" as utilized herein means screening for any activity of interest utilizing any fluorescent analyzer that detects fluorescence. Internal control reference genes which either express fluorescing molecules, such as those encoding green fluorescent protein, or encode proteins that can turnover fluorescing molecules, such as beta-galactosidase, can be utilized. These internal controls should optimally fluoresce at a wavelength which is different from the wavelength at which the molecule used to detect the evolved molecule(s) emits. DNA is evolved, recloned in a vector which co-expresses these proteins or molecules, transformed into an appropriate host organism, and rescreened utilizing the FACS machine to identify more desirable clones.

An important aspect of the invention is that cells are being analyzed individually. However other embodiments are contemplated which involve pooling of cells and multiple passage screen. This provides for a tiered analysis of biological activity from more general categories of activity, *i.e.* categories of enzymes, to specific activities of principle interest such as enzymes of that category which are specific to particular substrate molecules.

Members of these libraries can be encapsulated in gel microdroplets, exposed to substrates of interest, such as transition state analogs, and screened based on binding via FACS sorting for activities of interest.

It is anticipated with the present invention that one could employ mixtures of substrates to simultaneously detect multiple activities of interest simultaneously or sequentially. FACS instruments can detect molecules that fluoresce at different wavelengths, hence substrates which fluoresce at different wavelengths and indicate different activities can be employed.

The fluorescence activated cell sorting screening method of the present invention allows one to assay several million clones per hour for a desired bioactivity. This technique provides an extremely high throughput screening process necessary for the screening of extreme biodiverse environmental libraries.

In a preferred embodiment, the present invention provides a novel method for screening for activities, defined as "agents" herein, which affect the action of transducing proteins, such as, for example, G-proteins. In the present invention, cells containing

functional transducing proteins (such as membrane bound G-proteins), defined herein as "target cells" or "target(s)", are co-encapsulated with potential agent molecules and screened for affects agent molecules may have on their actions. Potential agent molecules are originally derived from a gene library generated from environmental or other samples, as described herein.

In particular, agents are molecules encoded by a pathway or gene cluster, or molecules generated by the expression of said pathways or clusters. Cells containing nucleic acid expressing the agent, or cells containing nucleic acid expressing activities which act within the cell to yield agent molecules can be utilized for screening. Alternatively, agent molecules can be expressed or generated prior to screening, and subsequently utilized. Cells expressing agent molecules, or agent molecules are coencapsulated, and screened utilizing various methods, such as those described herein.

Agent molecules can exist in or be introduced into the encapsulation particle by various means. Cells expressing genes encoding proteins which act to generate agent molecules (small molecules, for example) can be introduced into encapsulation particles using; for instance, Examples provided herein. Said cells can be prokaryotic or eukaryotic cells. Prokaryotic cells can be bacteria, such as *E.coli*. As previously indicated, genes can alternatively be expressed outside the encapsulation particle, the expression product or molecules generated via action of expressed products (such as small molecules or agent molecules) can be purified from the host, and said agents may be introduced into the encapsulation particle with the functional transducing protein(s), also using the methods described in the Examples below.

Encapsulation can be in beads, high temperature agaroses, gel microdroplets, cells, such as ghost red blood cells or macrophages, liposomes, or any other means of encapsulating and localizing molecules.

For example, methods of preparing liposomes have been described (*i.e.*, U.S. Patent No.'s 5,653,996, 5393530 and 5,651,981), as well as the use of liposomes to encapsulate a variety of molecules U.S. Patent No.'s 5,595,756, 5,605,703, 5,627,159, 5,652,225, 5,567,433, 4,235,871, 5,227,170). Entrapment of proteins, viruses, bacteria and DNA in erythrocytes during endocytosis has been described, as well (Journal of

Applied Biochemistry 4, 418-435 (1982)). Erythrocytes employed as carriers in vitro or in vivo for substances entrapped during hypo-osmotic lysis or dielectric breakdown of the membrane have also been described (reviewed in Ihler, G. M. (1983) J. Pharm. Ther). These techniques are useful in the present invention to encapsulate samples for screening.

"Microenvironment", as used herein, is any molecular structure which provides an appropriate environment for facilitating the interactions necessary for the method of the invention. An environment suitable for facilitating molecular interactions include, for example, liposomes. Liposomes can be prepared from a variety of lipids including phospholipids, glycolipids, steroids, long-chain alkyl esters; *e.g.*, alkyl phosphates, fatty acid esters; *e.g.*, lecithin, fatty amines and the like. A mixture of fatty material may be employed such a combination of neutral steroid, a charge amphiphile and a phospholipid. Illustrative examples of phospholipids include lecithin, sphingomyelin and dipalmitoylphos-phatidylcholine. Representative steroids include cholesterol, cholestanol and lanosterol. Representative charged amphiphilic compounds generally contain from 12-30 carbon atoms. Mono- or dialkyl phosphate esters, or alkyl amines; *e.g.*, dicetyl phosphate, stearyl amine; hexadecyl amine, dilauryl phosphate, and the like.

In addition, agents which potentially enhance or inhibit ligand/receptor interactions may be screened and identified. Thus, the present invention thus provides a method to screen recombinants producing drugs which block or enhance interactions of molecules, such as protein-protein interactions. When screening for compounds which affect G-protein interactions, host cells expressing recombinant clones to be screened are co-encapsulated with membrane bound G-proteins and ligands. Compounds (such as small molecules) diffuse out of host cells, and enhancement or inhibition of G-protein interactions can be evaluated via a variety of methods. Any screening method which allows one to detect an increase or decrease in activity or presence of an intracellular compound or molecule, including nucleic acids and proteins, which results from enhancement or inhibition of ligand/receptor interactions, transducers, such as G-protein interactions, or cascade events occurring inside a cell are useful in the present invention.

For example, the adenylyl cyclase method described above can be utilized in the present invention. Other assays which detect effects, or changes, modulated by effectors are useful in the present invention. The change, or signal, must be detectable against the background, or basal activity of the effector in the absence of the potential small molecule or drug. The signal may be a change in the growth rate of the cells, or other phenotypic changes, such as a color change or luminescence. Production of functional gene products may be impacted by the effect, as well. For example, the production of a functional gene product which is normally regulated by downstream or direct effects created by the transducer or effector can be altered and detected. Said functional genes may include reporter molecules, such as green fluorescent protein, or red fluorescent protein (Biosci Biotechnol Biochem 1995 Oct; 59(10):1817-1824), or other detectable molecules. These "functional genes" are used as marker genes. "Marker genes" are engineered into the host cell where desired. Modifications to their expression levels causes a phenotypic or other change which is screenable or selectable. If the change is selectable, a phenotypic change creates a difference in the growth or survival rate between cells which express the marker gene and those which do not, or a detectable modification in expression levels of reporter molecules within or around cells. If the change is screenable, the phenotype change creates a difference in some detectable characteristic of the cells, by which the cells which express the marker may be distinguished from those which do not. Selection is preferable to screening.

Rapid assays which measure direct readouts of transcriptional activity are useful in the present invention. For example, placing the bacterial gene encoding lacZ under the control of the FUS1 promoter, activation of the yeast pheromone response pathway can be detected in less than an hour by monitoring the ability of permeabilized yeast to produce color from a chromogenic substrate. Activation of other response pathways may be assayed via similar strategies. Genes encoding detectable molecules, or which create a detectable signal via modification of another molecules, can be utilized to analyze activation or suppression of a response.

The use of fluorescent proteins and/or fluorescent groups and quenching groups in close proximity to one another to assay the presence of enzymes or nucleic acid

sequences has been reported (WO 97/28261 and WO 95/13399). In the first of these reactions, fluorescent proteins having the proper emission and excitation spectra are put in physically close proximity to exhibit fluorescence energy transfer. Substrates for enzyme activities are placed between the two proteins, such that cleavage of the substrate by the presence of the enzymatic activity separates the proteins enough to change the emission spectra. Another group utilizes a fluorescent protein and a quencher molecule in close proximity to exhibit "collisional quenching" properties whereby the fluorescence of the fluorescent protein is diminished simply via the proximity of the quenching group. Probe nucleic acid sequences are engineered between the two groups, and a hybridization event between the probe sequence and a target in a sample separates the protein from the quencher enough to yield a fluorescent signal. Still another group has reported a combination of the above strategies, engineering a molecule which utilizes an enzyme substrate flanked by a fluorescent protein on one end and a quencher on the other (EP 0 428 000). It is recognized that these types assays can be employed in the method of the present invention to detect modifications in nucleic acid production (transcriptional activation or repression) and/or enzyme or other protein production (translational modifications) which results from inhibition of or improved association of interacting molecules, such as ligands and receptors, or which results from actions of bioactive compounds directly on transcription of particular molecules.

Fluorescent proteins encoded by genes which can be used to transform host cells and employed in a screen to identify compounds of interest are particularly useful in the present invention. Substrates are localized into the encapsulation means by a variety of methods, including but not limited to the method described herein in the Example below. Cells can also be engineered to contain genes encoding fluorescing molecules. For example, transcriptionally regulated genes can be linked to reporter molecule genes to allow expression (or lack of expression) of the reporter molecule to facilitate detection of the expression of the transcriptionally regulated gene. For example, if the ultimate effect of an agonist or antagonist interacting to enhance or inhibit the binding of a ligand to a receptor, or to enhance or inhibit the effects of any molecule in a pathway, is transcriptional activation or repression of a gene of interest the cell, it

is useful to be able to link the activated gene to a reporter gene to facilitate detection of the expression.

Cells can be engineered in variety of ways to allow the assay of the effect of compounds on cellular "events". An "event", as utilized herein, means any cellular function which is modified or event which occurs in response to exposure of the cell, or components of the cell, to molecules expressed by, or ultimately yielded by the expression of, members of gene libraries derived from samples and generated according to the methods described herein. For example, cellular events which can be detected with commercially available products include changes in transmembrane pH (*i.e.*, BCECF pH indicator sold by BioRad Laboratories, Inc., Hercules, California), cell cycle events, such as cell proliferation, cytotoxicity and cell death (*i.e.*, propidium iodide, 5-bromo-2'-deoxy-uridine (BrdU), Annexin-V-FLUOS, and TUNEL (method) sold by Boehringer-Mannheim Research Biochemicals), or production of proteins, such as enzymes. In many instances, the cascade of events begun by membrane protein interactions with other molecules involves modifications, such as phosphorylation or dephosphorylation, of molecules within the cell. Molecules, such as fluorescent substrates, which facilitate detection of these events are useful in the present invention to screen libraries expressing activities of interest. ELISA or colorimetric assays can also be adapted to single cell screening to be utilized to screen libraries according to the present invention.

Probe nucleic acid sequences designed according to the method described above can also be utilized in the present invention to "enrich" a population for desirable clones. "Enrich", as utilized herein, means reducing the number and/or complexity of an original population of molecules. For example, probes are designed to identify specific polyketide sequences, and utilized to enrich for clones encoding polyketide pathways. Figure X depicts in-situ hybridization of encapsulated fosmid clones with specific probes of interest, in this case polyketide synthase gene probes. Fosmid libraries are generated in E.coli according to the methods described in the Example herein. Clones are encapsulated and grown to yield encapsulated clonal populations. Cells are lysed and neutralized, and exposed to the probe of interest. Hybridization yields a

positive fluorescent signal which can be sorted on a fluorescent cell sorter. Positives can be further screened via expression, or activity, screening. Thus, this aspect of the present invention facilitates the reduction of the complexity of the original population to enrich for desirable pathway clones. These clones can the be utilized for further downstream screening. For example, these clones can be expressed to yield backbone structures (defined herein), which can the be decorated in metabolically rich hosts, and finally screened for an activity of interest. Alternatively, clones can be expressed to yield small molecules directly, which can be screened for an activity of interest. Further more, multiple probes can be designed and utilized to allow "multiplex" screening and/or enrichment. "Multiplex" screening and/or enrichment as used herein means that one is screening and/or enriching for more than desirable outcome, simultaneously.

Detectable molecules may be added as substrates to be utilized in screening assays, or genes encoding detectable molecules may be utilized in the method of the present invention.

The present invention provides for strategies to utilize high throughput screening mechanisms described herein to allow for the enrichment for desirable activities from a population of molecules. In one aspect of the present invention, cells are screened for the presence of ubiquitous molecules, such as thioesterase activities, to allow one to enrich for cells producing desirable bioactivities, such as those encoded by polyketide pathways. A variety of screening mechanisms can be employed. For example, identifying and recovering cells possessing thioesterase activities allows one to enrich for cells potentially containing polyketide activities. For example, for aromatic polyketides, the polyketide synthase consists of a single set of enzyme activities, housed either in a single polypeptide chain (type 1) or on separate polypeptides (type II), that act in every cycle. In contrast, complex polyketides are synthesized on multifunctional PKSs that contain a distinct active site for every catalyzed step in chain synthesis. Type I polyketide scaffolds are generated and cleaved from the acyl carrier protein in a final action by a thioesterase-cylcase activity (thioester bond cleaved). One group has even demonstrated that moving the location of the thioester bond along a polyketide pathway clone dictates where the polyketide scaffold will be clipped from the carrier protein

(Cortes J., et. al., Science, Vol. 258, 9 June 1995). Hybridization (homology) screening can be employed to identify cells containing thioesterase activities. If hybridization screening is utilized, sequences (partial or complete) of genes encoding known thioesterases can be utilized as identifying probes. Alternatively, probes containing probing sequences derived from known thioesterase activity genes, flanked by fluorescing molecules and/or quenching molecules, such as those described above, can be utilized. Labeled substrates can also be utilized in screening assays.

In another aspect of the present invention, screening using a fluorescent analyzer which requires single cell detection, such as a FACS machine, is utilized as a high throughput method to screen specific types of filamentous bacteria and fungi which form myceliates, such as Actinomyces or Streptomyces. In particular, screening is performed on filamentous fungi and bacteria which have, at one stage of their life cycle, unicells or monocells (multinucleoid cells fragment to produce monocells). Typically, spores of myceliate organisms germinate to make substrate mycelia (during which phase antibiotics are potentially produced), which then form arial mycelia. Arial mycelia eventually fragment to make more spores. Any filamentous bacteria or fungi which forms monocells during one stage of its life cycle can be screened for an activity of interest. Previously, this was not done because a branching network of multinucleoid (fungal like) cells forms with certain species. In a preferred embodiment, the present invention presents a particular species, Streptomyces venezuelae, for screening utilizing a fluorescent analyzer which requires single cell detection. The method of the present invention allows one to perform high throughput screening of myceliates for production of, for example, novel small molecules and bioactives. These cell types can be recombinant or non-recombinant.

Streptomyces venezuelae, unlike most other *Streptomyces* species, has been shown to sporulate in liquid grown culture. In some media, it also fragments into single cells when the cultures reach the end of vegetative growth. Because the production of most secondary metabolites, including bioactive small molecules, occurs at the end of log growth, it is possible to screen for *Streptomyces venezuelae* fragmented cells that are

producing bioactives by a fluorescence analyzer, such as a FACS machine, given the natural fluorescence of some small molecules.

In one aspect of the present invention, any *Streptomyces* or *Actinomyces* species that can be manipulated to produce single cells or fragmented mycelia is screened for a characteristic of interest. It is preferable to screen cells at the stage in their life cycle when they are producing small molecules for purposes of the present invention.

A fluorescence-based method for the selection of recombinant plasmids has been reported (BioTechniques 19:760-764, November 1995). *Escherichia coli* strains containing plasmids for the overexpression of the gene encoding uroporphyrinogen III methyltransferase accumulate fluorescent porphyrinoid compounds, which, when illuminated with ultraviolet light, causes recombinant cells to fluoresce with a bright red color. Replacement or disruption of the gene with other DNA fragments results in the loss of enzymatic activity and nonfluorescent cells.

Uroporphyrinogen III methyltransferase is an enzyme that catalyzes the S-adenosyl-l-methionine (SAM). -dependent addition of two methyl groups to uroporphyrinogen III methyltransferase to yield dihydrosirohydro-chlorin necessary for the synthesis of siroheme, factor F430 and vitamin B12. The substrate for this enzyme, uroporphyrinogen III (derived from ÿ-aminolevulinic acid) is a ubiquitous compound found not only in these pathways, but also in the pathways for the synthesis of the other so-called "pigments of life", heme and chlorophyll. Dihydrosirohydrochlorin is oxidated in the cell to produce a fluorescent compound sirohydochlorin (Factor II) or modified again by uroporphyrinogen III methyltransferase to produce trimethylpyrrocorphin, another fluorescent compound. These fluorescent compounds fluoresce with a bright red to red-orange color when illuminated with UV light (300nm).

Bacterial uroporphyrinogen III methylases have been purified from *E.coli* (1), *Pseudomonas* (2), *Bacillus* (3) and *Methanobacterium* (4). A *Bacillus stearothermophilus* uroporphyrinogen III methylase has been cloned sequenced and expressed in E.coli (Biosci Biotechnol Biochem 1995 Oct; 59(10):1817-1824).

In the method of the present invention, the fluorescing properties of this and other similar compounds can are utilized to screen for compounds of interest, as

described previously, or are utilized to enrich for the presence of compounds of interest. Host cells expressing recombinant clones potentially encoding gene pathways are screened for fluorescing properties. Thus, cells producing fluorescent proteins or metabolites can be identified. Pathway clones expressed in E.coli or other host cells, can yield bioactive compounds or "backbone structures" to bioactive compounds (which can subsequently be "decorated" in other host cells, for example, in metabolically rich organisms). The "backbone structure" is the fundamental structure that defines a particular class of small molecules. For example, a polyketide backbone will differ from that of a lactone, a glycoside or a peptide antibiotic. Within each class, variants are produced by the addition or subtraction of side groups or by rearrangement of ring structures ("decoration" or "decorated"). Ring structures present in aromatic bioactive compounds are known in some instance to yield a fluorescent signal, which can be utilized to distinguish these cells from the population. Certain of these structures can also provide absorbance characteristics which differ from the background absorbance of a non-recombinant host cell, and thus can allow one to distinguish these cells from the population, as well. Recombinant cells potentially producing bioactive compounds or "backbone" structures can be identified and separated from a population of cells, thus enriching the population for desirable cells. Thus, the method of the present invention also facilitates the discovery of novel aromatic compounds encoded by gene pathways, for example, encoded by polyketide genes, directly from environmental or other samples.

Compounds can also be generated via the modification of host porphyrin-like molecules by gene products derived from these samples. Thus, one can screen for recombinant clone gene products which modify a host porphyrin-like compound to make it fluoresce.

In yet another aspect of the present invention, cells expressing molecules of interest are sorted into 96-well or 384-well plates, specifically for further downstream manipulation and screening for recombinant clones. In this aspect of the present invention, the a fluorescence analyzer, such as a FACS machine is employed not to distinguish members of and evaluate populations or to screen as previously published, but to screen and recover positives in a manner that allows further screens to be

performed on samples selected. For example, typical stains used for enumeration can affect cell viability, therefore these types of stains were not employed for screening and selecting for further downstream manipulation of cells, specifically for the purpose, for example, of recovering nucleic acid which encodes an activity of interest. In particular, cells containing recombinant clones can be identified and sorted into multi-well plates for further downstream manipulation. There are various ways of screening for the presence of a recombinant clone in a cell. Genes encoding fluorescent proteins, such as green fluorescent protein (Biotechniques 19(4):650-655, 1995), or the gene encoding uroporphyrinogen III methyltransferase (BioTechniques 19:760-764, November 1995) can be utilized in the method of the present invention as reporters to allow detection of recombinant clones. Recombinant clones are sorted for further downstream screening for an activity of interest. Screening may be for an enzyme, for example, or for a small molecule, and may be performed using any variety of methods, including those described or referred to herein.

In yet another aspect of the present invention, desirable existing compounds are modified, and evaluated for a more desirable compound. Existing compounds or compound libraries are exposed to molecules generated via the expression of small or large insert libraries generated in accordance with the methods described herein. Desirable modifications of these existing compounds by these molecules are detected and better lead compounds are screened for utilizing a fluorescence analyzer, such as a FACS machine. For example, E. coli cells expressing clones yielding small molecules are exposed to one or more existing compounds, which are subsequently screened for desirable modifications. Alternatively, cells are co-encapsulated with one or more existing compounds, and screened simultaneously to identify desirable modifications to the compound. Examples of modifications include covalent or non-covalent modifications. Covalent modifications include incorporation, transfer and cleavage modifications, such as the addition or transfer of methyl groups or phosphate groups to a compound, or the cleavage of a peptide or other bond to yield an active compound. Non-covalent modifications include conformational changes made to a molecule via addition or disruption of, for example, hydrogen bonds, ionic bonds, and/or Van der Wals

forces. Modified compounds can be screened by various means, including those described herein.

Alternatively, existing compounds are utilized to modify the molecules generated via the expression of large or small insert clones, and desirable modifications of the molecules are screened for via fluorescence screening, utilizing various methods, including those described herein.

In another aspect of the present invention, molecules derived from expressed clones are exposed to organisms to enrich for potential compounds which cause growth inhibition or death of cells. For example, cultures of Staphylococcus aureus are co-encapsulated with compounds generated via expression of clones, or with cells expressing clones, and allowed to grow for a period of time by exposure to select media. Co-encapsulated products are then stained and screened for via fluorescence screening. Stains which allow detection of live cells can be utilized, allowing positives, which in this case would have no fluorescence, to be recovered. Alternatively, forward and side scatter characteristics are used to enrich for positives. Less or no growth of Staphylococus or other organisms being evaluated will yield capsules with less forward and/or side scatter.

In another aspect of the present invention clones expressing useful bioactivities are screened in-vivo. In this aspect, host cells are stimulated to internalize recombinant cells, and used to screen for bioactivities generated by these recombinant cells which can cause host cell death or modify an internal molecule or compound within the host.

Many bacterial pathogens survive in phagocytes, such as macrophages, by coordinately regulating the expression of a wide spectrum of genes. A microbes ability to survive killing by phagocytes correlates with its ability to cause disease. Hence, the identification of genes that are preferentially transcribed in the intracellular environment of the host is central to understanding of how pathogenic organisms mount successful infection.

Valdivia and Falkow have reported a selection methodology to identify genes from pathogenic organisms that are induced upon association with host cells or tissues.

The group noted that fourteen Salmonella typhimuium genes, under control of at least four independent regulatory circuits, were identified to be selectively induced in host macrophages. The methodology is based on differential fluorescence induction (DFI) for the rapid identification of bacterial genes induced upon association with host cells that would work independently of drug susceptibility and nutritional requirements.

Differential fluorescence induction is employed in one aspect of the present invention to screen macrophages harboring bacterial clones carrying any virulence gene fused to a reporter molecule and a clone of a putative bioactive pathway. Macrophage cells are coinfected in the method of the present invention with clones of pathways potentially encoding useful bioactives, and plasmids or other vectors encoding virulence factors. Thus, one aspect of the present invention allows one to screen recombinant bioactive molecules that inhibit transcriptionally active reporter gene fusions in macrophage or other phagocyte cells. Bioactive molecules which inhibit virulence factors in-vivo are identified via a lack of expression of the reporter molecule, for example red or green fluorescent proteins. This method allows for the rapid screening for pathways encoding bioactive compounds specifically inhibiting a virulence factor or other gene product. Thus the screen allows one to identify biologically relevant molecules active in mammalian cells.

Recombinant bioactive compounds can also be screened *in vivo* using "two-hybrid" systems, which can detect enhancers and inhibitors of protein-protein or other interactions such as those between transcription factors and their activators, or receptors and their cognate targets. Figure 7 depicts an approach to screen for small molecules that enhance or inhibit transcription factor initiation. Both the small molecule pathway and the GFP reporter construct are co-expressed. Clones altered in GFP expression can then be sorted by FACS and the pathway clone isolated for characterization.

As indicated, common approaches to drug discovery involve screening assays in which disease targets (macromolecules implicated in causing a disease) are exposed to potential drug candidates which are tested for therapeutic activity. In other approaches, whole cells or organisms that are representative of the causative agent of the

disease, such as bacteria or tumor cell lines, are exposed to the potential candidates for screening purposes. Any of these approaches can be employed with the present invention.

The present invention also allows for the transfer of cloned pathways derived from uncultivated samples into metabolically rich hosts for heterologous expression and downstream screening for bioactive compounds of interest using a variety of screening approaches briefly described above.

Recovering Desirable Bioactivities

After viable or non-viable cells, each containing a different expression clone from the gene library are screened, and positive clones are recovered, DNA is isolated from positive clones utilizing techniques well known in the art. The DNA can then be amplified either *in vivo* or *in vitro* by utilizing any of the various amplification techniques known in the art. *In vivo* amplification would include transformation of the clone(s) or subclone(s) of the clones into a viable host, followed by growth of the host. *In vitro* amplification can be performed using techniques such as the polymerase chain reaction.

Evolution

One advantage afforded by a recombinant approach to the discovery of novel bioactive compounds is the ability to manipulate pathway subunits to generate and select for variants with altered specificity. Pathway subunits can be substituted or individual subunits can be evolved utilizing methods described below, to select for resultant bioactive molecules with different activities.

Clones found to have the bioactivity for which the screen was performed can be subjected to directed mutagenesis to develop new bioactivities with desired properties or to develop modified bioactivities with particularly desired properties that are absent or less pronounced in the wild-type activity, such as stability to heat or organic solvents. Any of the known techniques for directed mutagenesis are applicable to the invention.

For example, particularly preferred mutagenesis techniques for use in accordance with the invention include those described below.

The term "error-prone PCR" refers to a process for performing PCR under conditions where the copying fidelity of the DNA polymerase is low, such that a high rate of point mutations is obtained along the entire length of the PCR product. Leung, D.W., *et al.*, Technique, 1:11-15 (1989) and Caldwell, R.C. & Joyce G.F., PCR Methods Applic., 2:28-33 (1992).

The term "oligonucleotide directed mutagenesis" refers to a process which allows for the generation of site-specific mutations in any cloned DNA segment of interest. Reidhaar-Olson, J.F. & Sauer, R.T., *et al.*, Science, 241:53-57 (1988).

The term "assembly PCR" refers to a process which involves the assembly of a PCR product from a mixture of small DNA fragments. A large number of different PCR reactions occur in parallel in the same vial, with the products of one reaction priming the products of another reaction.

The term "sexual PCR mutagenesis" (also known as "DNA shuffling") refers to forced homologous recombination between DNA molecules of different but highly related DNA sequence *in vitro*, caused by random fragmentation of the DNA molecule based on sequence homology, followed by fixation of the crossover by primer extension in a PCR reaction. Stemmer, W.P., PNAS, USA, 91:10747-10751 (1994).

The term "*in vivo* mutagenesis" refers to a process of generating random mutations in any cloned DNA of interest which involves the propagation of the DNA in a strain of *E. coli* that carries mutations in one or more of the DNA repair pathways. These "mutator" strains have a higher random mutation rate than that of a wild-type parent. Propagating the DNA in one of these strains will eventually generate random mutations within the DNA.

The term "cassette mutagenesis" refers to any process for replacing a small region of a double stranded DNA molecule with a synthetic oligonucleotide "cassette" that differs from the native sequence. The oligonucleotide often contains completely and/or partially randomized native sequence.

The term "recursive ensemble mutagenesis" refers to an algorithm for protein engineering (protein mutagenesis) developed to produce diverse populations of phenotypically related mutants whose members differ in amino acid sequence. This method uses a feedback mechanism to control successive rounds of combinatorial cassette mutagenesis. Arkin, A.P. and Youvan, D.C., PNAS, USA, 89:7811-7815 (1992).

The term "exponential ensemble mutagenesis" refers to a process for generating combinatorial libraries with a high percentage of unique and functional mutants, wherein small groups of residues are randomized in parallel to identify, at each altered position, amino acids which lead to functional proteins, Delegrave, S. and Youvan, D.C., Biotechnology Research, 11:1548-1552 (1993); and random and site-directed mutagenesis, Arnold, F.H., Current Opinion in Biotechnology, 4:450-455 (1993).

The use of a culture-independent approach to directly clone genes encoding novel enzymes from environmental samples allows one to access untapped resources of biodiversity. The approach is based on the construction of "environmental libraries" which represent the collective genomes of naturally occurring organisms archived in cloning vectors that can be propagated in suitable prokaryotic hosts. Because the cloned DNA is initially extracted directly from environmental samples, the libraries are not limited to the small fraction of prokaryotes that can be grown in pure culture. Additionally, a normalization of the environmental DNA present in these samples could allow more equal representation of the DNA from all of the species present in the original sample. This can dramatically increase the efficiency of finding interesting genes from minor constituents of the sample which may be under-represented by several orders of magnitude compared to the dominant species.

In the evaluation of complex environmental expression libraries, a rate limiting step previously occurred at the level of discovery of bioactivities. The present invention allows the rapid screening of complex environmental expression libraries, containing, for example, thousands of different organisms. The analysis of a complex sample of this size requires one to screen several million clones to cover this genomic

biodiversity. The invention represents an extremely high-throughput screening method which allows one to assess this enormous number of clones. The method disclosed allows the screening anywhere from about 30 million to about 200 million clones per hour for a desired biological activity. This allows the thorough screening of environmental libraries for clones expressing novel biomolecules.

The present invention combines a culture-independent approach to directly clone genes encoding novel bioactivities from environmental samples with an extremely high throughput screening system designed for the rapid discovery of new biomolecules.

The strategy begins with the construction of gene libraries which represent the genome(s) of microorganisms archived in cloning vectors that can be propagated in *E. coli* or other suitable prokaryotic hosts. Preferably, "environmental libraries" which represent the collective genomes of naturally occurring microorganisms are generated. In this case, because the cloned DNA is extracted directly from environmental samples, the libraries are not limited to the small fraction of prokaryotes that can be grown in pure culture. In addition, "normalization" can be performed on the environmental nucleic acid as one approach to more equally represent the DNA from all of the species present in the original sample. Normalization techniques can dramatically increase the efficiency of discovery from genomes which may represent minor constituents of the environmental sample. Normalization is preferable since at least one study has demonstrated that an organism of interest can be underrepresented by five orders of magnitude compared to the dominant species.

In another embodiment the invention provides a device for the isolation and containment of microorganisms and a method for acquiring *in situ* enrichments of uncultivated microorganisms. The enrichment process can increase the likelihood of recovering rare species and previously uncultivated members of a microbial population.

In situ enrichment can be achieved in the present invention by using a microbial containment device consisting of growth substrates and nutritional amendments with the intent to selectively lure members of the surrounding environmental matrix. Choice of substrates (carbon sources) and nutritional amendments (i.e., nitrogen, phosphorous, etc.) is dependent upon the members of the community for

which one desires to enrich. The exact composition depends upon which members of the community one desires to enrich and which members of the community one desires to inhibit. These containment devices are then deployed in desired biotopes for a period of time to allow attraction and growth of desirable microbes.

Substrates of the invention can include monomers and polymers. Monomers of substrates, such as glucosamine, cellulose, pentanoic or other acids, xylan, chitin, etc., can be utilized for attraction of certain types of microbes. Using monomers allows one to depend on attraction for the collecting, versus the presence of substrate receptors on cells. This could provide the added benefit of allowing one to acquire more biodiversity. Polymers can also be used to attract microbes that can degrade them.

Specific microbes of interest can be captured and concentrated from dilute populations in aqueous environments thereby obviating the need to concentrate microorganisms from large volumes of water. These devices can also be implanted in soil environments to enrich microbes from terrestrial habitats. Substrates such as cellulose or chitin can be attached to the surface material to attract specific classes of microbes, such as the actinomyces, which are a rich source of secondary metabolites.

Utilizing the present invention, *in situ* enrichment can be readily achieved. Figure 2 demonstrates the capture of microbes from different habitats, as detailed in the present invention. These photos demonstrate the difference in the types of microbes collected from a soil environment when utilizing two different types of substrates (cellulose and xylan). These photos also demonstrate the difference in employing beads alone versus beads with substrate attached (chitin).

In a preferred embodiment, the invention relates to a microbial containment device for collecting a population of microorganisms from an environmental sample comprising a solid support having a surface for attaching a selectable microbial enrichment media.

In another preferred embodiment of the invention, a method for isolating microorganisms from an environmental sample comprising contacting the sample with a device having a solid support and a surface for attaching a selectable microbial enrichment media and isolating the population from the device is provided.

"Selective microbial enrichment media", as used herein, is any medium containing elements which enhance the growth of certain organisms and/or inhibit the growth of other organisms present in the surrounding environment. The media of the present invention is useful when the organism targeted for enrichment is present in relatively small numbers compared to other organisms growing in the surrounding matrix. For example, a selective microbial enrichment media containing the antibiotics colistin and nalidixic acid will inhibit the growth of gram-negative bacteria but not the growth of gram-positives. The selectivity of the microbial enrichment media can be further enhanced by the addition of a specific substrate such as, for example, cellulose, to the colistin and nalidixic acid containing media. Therefore, a microbial containment device incorporating the aforementioned microbial enrichment media will be selective for gram-positive organisms which are capable of utilizing cellulose as an energy source.

The term "solid support", as used herein, is any structure which provides a supporting surface for the attachment of a selectable microbial enrichment media. Well known solid supports that may be used for screening assays of the invention include, but are not restricted to, glass beads, silica aerogels, agarose, Sepharose, Sephadex, nitrocellulose, polyethylene, dextran, nylon, natural and modified cellulose, polyacrylamide, polystyrene, polypropylene, and microporous polyvinylidene difluoride membrane. It is understood that any material which allows for the attachment and support of a selectable media is included in the present invention. By using large surface area materials, such as, for example, glass beads or silica aerogels, a high concentration of microbes can be collected in a relatively small device holding multiple collections of substrate-surface conjugates.

In one aspect of the invention, substrates are conjugated to solid surfaces prior to deployment into the environment of choice. Such conjugation is preferably a chemical conjugation. Large surface area materials, such as glass beads or silica aerogels are preferably utilized as surfaces in the present invention. It is anticipated that there are a variety of surface area materials that could be utilized effectively in the present invention. Conjugation or immobilization of substrates to the surface material may occur via a variety of methods apparent to the skilled artisan. One example of derivitization

of glass beads is described in an Example provided below. It is anticipated that any of a variety of conjugation or immobilization strategies can be employed to immobilize substrates to surfaces in the present invention.

Derivitized surface area materials, such as glass beads or silica aerogels, of the present invention are contained in separate device(s) before placement into the environment of interest. Preferably, such containment devices are of the type which allow migration of microbes in while simultaneously containing the derivitized materials. For example, particularly preferred containers are mesh filters, such as those available from Spectrum in Houston, Texas, which have been manipulated to contain the derivitized materials. For example, filters can be cut into squares, derivitized materials can be placed in the center, the filter can be folded in half and the three sides can be glued shut to create a containment device. Mesh filters, or the like, can then be placed in any device to be used as a solid support which will contain the mesh filter for deployment into the environment. Particularly preferred devices are made of inert materials, such as plexiglass.

Alternatively, any device which allows migration of microbes while simultaneously containing the materials can be employed with the present invention. For example, Falcon tubes (VWR, Fisher Scientific) or the like may be employed to contain the derivitized materials directly. Said tubes can be punctured utilizing a sharp instrument to yield a device which allows microbe migration into or out of the device.

The anchored component of the selectable enrichment medium can be immobilized by non-covalent or covalent attachments. Non-covalent attachment can be accomplished by coating a solid surface with a solution of, for example, a protein which is specifically recognized by a receptor displayed on the cell membrane of a target organism. Alternatively, an immobilized antibody, preferably a monoclonal antibody, specific for the protein to be immobilized can be used to anchor the protein to the solid surface. The surfaces can be prepared in advance and stored.

In another aspect, the present invention relates to a method of selective *in situ* enrichment of bacterial and archaeal microorganisms utilizing a microbial attractant attached to a solid surface. A "microbial attractant", as used herein, is defined as any

composition which selectively precipitates or induces the migration of microorganisms to a device containing a microbial enrichment media. A microbial attractant is further defined as any composition which selectively augments the survival of a microorganism which contacts a microbial enrichment media contained in a device of the present invention. For example, microorganisms routinely display chemotactic responses to environmental stimuli perceived as energy sources, such as a carbon source. Any particular carbon source can be utilized by some members of the community and not others. Carbon source selection thus depends upon the members of the community one desires to enrich. For example, members of the *Streptomycetales* tend to utilize complex, polymeric substrates such as cellulose, chitin, and lignin. These complex subtrates, while utilized by other genera, are recalcitrant to most bacteria.

In another aspect, the use of additional nitrogen sources may be called for depending upon the choice for carbon source. For example, while chitin is balanced in its C:N ratio, cellulose is not. To enhance utilization of cellulose (or other carbon-rich substrates), it is often useful to add nitrogen sources such as nitrate or ammonia. Further, the addition of trace elements may enhance growth of some members of a community while inhibiting others. In another aspect of the invention, compounds useful as growth inhibitors of eukaryotic organisms can be included in the device of the present invention. Growth inhibitors of eukaryotic organisms include any compound which selectively prevents the growth of eukaryotic organisms. Such inhibitors can include, for example, one or more commercially available compounds such as nystatin, cycloheximide, and/or pimaricin or other antifungal compounds. These compounds may be sprinkled as a powder or incorporated as a liquid in the selectable microbial enrichment medium. It is anticipated that other selective agents can be employed to inhibit the growth of undesired species or promote the growth of desired species. For example, obtaining bacterial and archaeal species can be complicated by the presence of eukaryotic organisms which can out-compete desired bacterial species for the available substrate. Therefore, including selective agents, such as antifungal agents or other eukaryotic growth inhibitors, in the device of the present invention promotes the growth of target microorganisms.

In yet another aspect, compounds which inhibit the growth of some bacterial species, but not others, may be incorporated into the enrichment medium. Growth inhibitors for prokaryotic organisms include any compound which prevents the proliferation of prokaryotic cells. Such compounds include, but are not limited to, polymyxin, penicillin, and rifampin. Use of the compounds is dependent upon which members of the bacterial community one desires to enrich. For example, while a majority of the *Streptomyces* are sensitive to polymyxin, penicillin, and rifampin, these may be used to enrich for "rare" members of the family which are resistant. Selective agents may also be used in enrichments for archaeal members of the community.

In the context of the present invention, a containment device containing a microbial enrichment medium can incorporate, for example, a complex carbon source as an attractant, nystatin as an inhibitor of eukaryotic organisms and rifampin as an inhibitor of selected prokaryotic organisms. It is understood that attractants, eukaryotic inhibitors and prokaryotic inhibitors can be used individually, or in any combination, as a component of a selectable microbial enrichment medium of the present invention. It is further understood that a device of the present invention can include any appropriate solid support in combination with any microbial enrichment medium suitable for an environmental matrix or for the isolation of microorganisms of interest. An environmental matrix can include a marine environment, a terrestrial environment or a combination of marine and terrestrial environment. Moreover, an environmental matrix can include those organisms which exist in surroundings which are neither solid nor liquid, such as those organisms which remain airborne. The device of the present invention can be used to filter such organisms from the atmosphere or any other gaseous environment. It is further envisioned that a containment device of the present invention can be used for the isolation of microorganisms from non-terrestrial environments, such as those existing on planets other than earth. For example, a containment device containing a microbial enrichment medium designed to attract microorganisms which can exist on the planet Mars is included in the present invention. Such a device would incorporate features designed to attract microorganisms capable of existing in an environmental matrix not substantially different from those which are currently

encountered on earth. Further, a sufficient amount of data concerning environmental conditions on planets other than earth is available such that a containment device of the present invention can be designed to incorporate elements specific to those environments.

In another aspect, the present invention can be employed to isolate and identify microorganisms useful in bioremediation. Bioremediation is a process which utilizes microorganisms to remove or detoxify toxic unwanted chemicals from an environment. The device of the present invention can be modified to contain a medium which selectively enriches for those organisms capable of attaching to, or detoxifying, toxic or unwanted chemicals. For example, halogenated organic compounds have had widespread use as fungicides, herbicides, insecticides, algaecides, plasticizers, solvents, hydraulic fluids, refrigerants and intermediates for chemical syntheses. As a result, they constitute one of the largest groups of environmental pollutants. Chloroorganic compounds comprise the largest fraction of these materials, having been synthesized by large scale processes over the past few decades. Their ubiquitous use and distribution in our ecosystem has raised concern over their possible effects on public health and the environment. Therefore, a need exists for the identification of microorganisms which are capable of removing these, and other, chemicals from the environment. The inclusion, for example, of chlorinated organic compounds in a selectable enrichment medium of the present invention can aid the isolation of organisms attracted to such a compound. Other such compounds may include alkanes, aromatics, sulphonyls and heavy metals. Once identified, the organism can be used as a natural and inexpensive means of detoxifying environments known to contain such pollutants.

All of the references mentioned above are hereby incorporated by reference in their entirety. Each of these techniques is described in detail in the references mentioned. DNA can be mutagenized, or "evolved", utilizing any one or more of these techniques, and rescreened to identify more desirable clones. The invention will now be illustrated by the following working examples, which are in no way a limitation thereof.

Example 1

Sample Collection Using A Microbial Containment Device

Sample to be utilized for downstream nucleic acid isolation for library generation may be collected according to the following example:

The following represents a method of selective *in situ* enrichment of bacterial and archaeal species while at the same time inhibiting the proliferation of eukaryotic members of the population.

In situ enrichment is achieved by using ÒtrapsÓ composed of growth substrates and nutritional amendments with the intent to lure, selectively, members of the surrounding environmental matrix, coated onto surfaces. Choice of substrates (carbon sources) and nutritional amendments (ie, nitrogen, phosphorous, etc.) is dependent upon the members of the community one desires to enrich. Selective agents against eukaryotic members are also added to the trap. Again, the exact composition will depend upon which members of the community one desires to enrich and which members of the community one desires to inhibit. Substrates include monomers and polymers. Monomers of substrates, such as glucosamine, cellulose, pentanoic or other acids, xylan, chitin, etc., can be utilized for attraction of certain types of microbes. Polymers can also be used to attract microbes that can degrade them. Some of the enrichment ÒmediaÓ which may be useful in pulling out particular members of the community is described below:

1. Addition of bioactive compounds against fungi and microscopic eukaryotes:

Proliferation of eukaryotic members of the community may be inhibited by the use of one or more commercially available compounds such as nystatin, cycloheximide, and/or pimaricin. These compounds may be sprinkled as a powder or incorporated as a liquid in the selective enrichment medium.

2. Addition of bioactive compounds against other bacterial species:

Compounds which inhibit the growth of some bacterial species but not others (ie, polymyxin, penicillin, and rifampin) may be incorporated into the enrichment medium. Use of the compounds is dependent upon which members of the bacterial community one desires to enrich. For example, while a majority of the *Streptomyces* are sensitive to polymyxin, penicillin, and rifampin, these may be used to enrich for ÒrareÓ members of the family which are resistant. Selective agents may also be used in enrichments for archaeal members of the community.

3. Use of carbon sources as selective agents:

Any particular carbon source can be utilized by some members of the community and not others. Carbon source selection thus depends upon the members of the community one desires to enrich. For example, members of the *Streptomycetales* tend to utilize complex, polymeric substrates such as cellulose, chitin, and lignin. These complex subtrates, while utilized by other genera, are recalcitrant to most bacteria. These complex substrates are utilized by fungi, which necessitates the use of anti-fungal agents, mentioned above.

4. Addition of nitrogen sources:

The use of additional nitrogen sources may be called for depending upon the choice for carbon source. For example, while chitin is balanced in its C:N ratio, cellulose is not. To enhance utilization of cellulose (or other carbon-rich substrates), it is often useful to add nitrogen soures such as nitrate or ammonia.

5. Addition of trace elements:

In general, the environmental matrix tends to be a good source of trace elements, but in certain environments, the elements may be limiting. Addition of trace elements may enhance growth of some members of the community while inhibiting others.

Large surface area materials, such as glass beads or silica aerogels can be utilized as surfaces in the present example. This allows a high concentration of microbes to be collected in a relatively small device holding multiple collections of substrate-surface conjugates.

Glass beads can be derivitized with N-Acetyl B- D -glucosamine-phenylisothiocyanate as follows:

Bead Preparation:

30ml glass beads (Biospec Products, Bartlesville, OK) are mixed with 50ml APS/Toluene (10% APS) (Sigma Chemical Co.)

Reflux overnight

Decant and wash 3 times with Toluene

Wash 3 times with ethanol and dry in oven

Derivitize with N-Acetyl B- D -glucosamine-phenylisothiocyanate as follows:

Combine in Falcon Tube:

25 ml prepared glass beads from above

15 ml 0.1M $NaHCO_3$ + 25mg N-Acetyl-B-D-glucosamine-PITC (Sigma Chemical Co.) + 1 ml DMSO

Add 10ml $NaHCO_3$ + 1 ml DMSO

Pour over glass beads

Let shake in Falcon Tube overnight

Wash with 20ml 0.1M $NaHCO_3$

Wash with 50ml ddH_2O

Dry at 55°C for 1 hour

Beads can then be placed in mesh filter "bags" (Spectrum, Houston, Texas) created to allow containment of the beads, while simultaneously allowing migration of microbes, which are then placed in any device used as a solid support which allows containment of the bag. Particularly preferred devices are made of inert materials, such as plexiglass. Alternatively, beads can be placed directly into Falcon Tubes (VWR, Fisher Scientific)

which have been punctured with holes using a needle. These "containment" devices are then deployed in desired biotopes for a period of time to allow attraction and growth of desirable microbes.

The following protocol details one method for generating a simple "microbial containment device":

Puncture holes using a heated needle or other pointed device into a 15ml Falcon Tube (VWR, Fisher Scientific).

Place approximately 1-5mls of the derivitized beads into a Spectra/mesh nylon filter, such as those available from Spectrum (Houston, Texas) with a mesh opening of 70 m, an open area of 43%, and a thickness of 70 m. Seal the nylon filter to create a "bag" containing the beads using, for instance, Goop, Houshold Adhesive & Sealant.

Place the filter containing the beads into the ventilated Falcon Tube and deploy the tube into the desired biotope for a period of time (typically days).

Example 2

DNA Isolation and Library Construction from Cultivated Organism

The following outlines the procedures used to generate a gene library from an isolate, *Streptomyces rimosus*.

Isolate DNA.

1. Inoculate 25ml Trypticase Soy Broth (BBL Microbiology Systems) in 250 ml baffled erlenmeyer flasks with spores of Streptomyces rimosus. Incubate at 30°C at 250rpm for 48 hours.
2. Collect mycelin by centrifugation. Use 50ml conical tubes and centrifuge at 25°C at 4000rpm for 10 minutes.
3. Decant supernatent and wash pellet 2X with 10 ml 10.3% sucrose (centrifuge as above between washes).

4. Store pellet at -20°C for future use.

5. Resuspend pellet in 40ml TE (10mM Tris, 1mM EDTA; pH 7.5) containing lysozyme (1mg/ml; Sigma Chemical Co.)and incubate at 37°C for 45 minutes.

6. Add sarcosyl (N-lauroylsarcosine, sodium salt, Sigma Chemical Co.) to final concentration of 1% and invert gently to mix for several minutes.

7. Transfer 20ml of preparation to clean tube and add proteinase K (Stratagene Cloning Systems) to a final concentration of 1mg/ml. Incubate overnight at 50°C.

8. Extract 2X with Phenol (saturated with TE).

9. Extract 1X with Phenol:CH_3Cl.

10. Extract 1X with CH_3Cl: Isoamyl alcohol.

11. Precipitate DNA with 2 volumes of EtOH.

12. Spool DNA on sealed pasteur pipet.

13. Rinse with 70% EtOH.

14. Dry in air.

15. Resuspend DNA in 1ml TE and store at 4°C to rehydrate slowly.

16. Check quality of DNA:
Digest 10 ml DNA with EcoRI restriction enzyme (Stratagene Cloning Systems) according to manufacturers protocol electrophorese DNA digest through 0.5% agarose, 20V overnight; stain gel in 1 g/ml EtBr

17. Determine DNA concentration (A_{260}-A_{280}).

Restriction Digest DNA

1. Incubate the following at 37°C for 3 hours:

8 ml DNA (~10 mg)
35 ml H_2O
5 ml 10x restriction enzyme buffer
2 ml EcoRI restriction enzyme (200 units)

Sucrose Gradient

1. Prepare small sucrose gradient (Sambrook, Fritsch and Maniatus, 1989) and run DNA at 45,000 rpm for 4 hours at 25°C.
2. Examine 5 ml of each fraction on 0.8% agarose gel.
3. Pool relevant fractions and precipitate DNA with 2.5 volumes of EtOH for 1 hour at -70°C.
4. Collect DNA by centrifugation at 13,200 rpm for 15 minutes.
5. Decant and wash with 1ml of 70% EtOH.
6. Dry, resuspend in 15 ml TE.
7. Store at 4°C.

Dephosphorylate DNA

1. Dephosphorylate DNA with shrimp alkaline phosphatase according to manufacturers protocol (US Biochemicals).

Adaptor Ligation

1. Ligate adaptors according to manufacturers protocol.
2. Briefly, gently resuspend DNA in EcoR I-BamH I adaptors (Stratagene Cloning Systems); add 10X ligation buffer, 10mM rATP, and T4 DNA ligase and incubate at room temperature for 4-6 hours.

Preparation of Fosmid Arms

1. Fosmid arms can be prepared as described (Kim, et.al., Nucl. Acids Res., 20:10832-10835, 1992). Plasmid DNA can be digested with PmeI restriction enzyme (New England Biolabs) according to the manufacturers protocol, dephosphorylated (Sambrook, Fritsch and Maniatus, 1989), followed by a digestion with BamH I restriction enzyme (New England Biolabs) according to the manufacturers protocol, and another dephosphorylation step to generate

two arms each of which contain a cos site in the proper orientation for the cloning and packaging of ligated DNA between 35-45 kbp.

Ligation to Fosmid Arms

1. Prepare the ligation reaction:
   Add ~50ng each of insert and vector DNA to 1U of T4 DNA ligase (Boehringer Mannheim) and 10X ligase buffer as per manufacturers instructions; add H_2O if necessary, to total of 10 ml.
2. Incubate overnight at 16°C.

Package and Plate

I. Package the ligation reactions using Gigapack XL packaging system (Stratagene Cloning Systems, Inc.) following manufacturer's protocol.

2 Transfect E.coli strain DH10B (Bethesda Research Laboratories, Inc.) according to manufacturers protocol and spread onto LB/Chloramphenicol plates (Sambrook, Fritsch and Maniatus, 1989).

Example 3

Preparation of an Uncultivated Prokaryotic DNA Library

Figure 1 shows an overview of the procedures used to construct an environmental library from a mixed picoplankton sample. The goal was to construct a stable, large insert DNA library representing picoplankton genomic DNA.

Cell collection and preparation of DNA. Agarose plugs containing concentrated picoplankton cells were prepared from samples collected on an oceanographic cruise from Newport, Oregon to Honolulu, Hawaii. Seawater (30 liters) was collected in Niskin bottles, screened through 10 mm Nitex, and concentrated by hollow fiber filtration (Amicon DC10) through 30,000 MW cutoff polysulfone filters. The concentrated bacterioplankton cells were collected on a 0.22 mm, 47 mm Durapore

filter, and resuspended in 1 ml of 2X STE buffer (1M NaCl, 0.1M EDTA, 10 mM Tris, pH 8.0) to a final density of approximately 1 X 10^{10} cells per ml. The cell suspension was mixed with one volume of 1% molten Seaplaque LMP agarose (FMC) cooled to 40°C, and then immediately drawn into a 1 ml syringe. The syringe was sealed with parafilm and placed on ice for 10 min. The cell-containing agarose plug was extruded into 10 ml of Lysis Buffer (10mM Tris pH 8.0, 50 mM NaCl, 0.1M EDTA, 1% Sarkosyl, 0.2% sodium deoxycholate, a mg/ml lysozyme) and incubated at 37°C for one hour. The agarose plug was then transferred to 40 mls of ESP Buffer (1% Sarcosyl, 1 mg/ml proteinase-K, in 0.5M EDTA), and incubated at 55°C for 16 hours. The solution was decanted and replaced with fresh ESP Buffer, and incubated at 55°C for an additional hour. The agarose plugs were then placed in 50 mM EDTA and stored at 4°C shipboard for the duration of the oceanographic cruise.

One slice of an agarose plug (72 ml) prepared from a sample collected off the Oregon coast was dialyzed overnight at 4°C against 1 ml of buffer A (100mM NaCl, 10mM Bis Tris Propane-HCl, 100 g/ml acetylated BSA: pH 7.0 @ 25°C) in a 2 ml microcentrifuge tube. The solution was replaced with 250 l of fresh buffer A containing 10 mM $MgCl_2$ and 1 mM DTT and incubated on a rocking platform for 1 hr at room temperature. The solution was then changed to 250 ml of the same buffer containing 4U of Sau3A1 (NEB), equilibrated to 37°C in a water bath, and then incubated on a rocking platform in a 37°C incubator for 45 min. The plug was transferred to a 1.5 ml microcentrifuge tube and incubated at 68°C for 30 min to inactivate the protein, *e.g.* enzyme, and to melt the agarose. The agarose was digested and the DNA dephosphorylased using Gelase and HK-phosphatase (Epicentre), respectively, according to the manufacturer's recommendations. Protein was removed by gentle phenol/chloroform extraction and the DNA was ethanol precipitated, pelleted, and then washed with 70% ethanol. This partially digested DNA was resuspended in sterile H_2O to a concentration of 2.5 ng/ l for ligation to the pFOS1 vector.

PCR amplification results from several of the agarose plugs (data not shown) indicated the presence of significant amounts of archaeal DNA. Quantitative hybridization experiments using rRNA extracted from one sample, collected at 200 m of

depth off the Oregon Coast, indicated that planktonic archaea in (this assemblage comprised approximately 4.7% of the total picoplankton biomass (this sample corresponds to "PACI"-200 m in Table 1 of DeLong *et al.*, high abundance of Archaea in Antarctic marine picoplankton, *Nature, 371*:695-698, 1994). Results from archaeal-biased rDNA PCR amplification performed on agarose plug lysates confirmed the presence of relatively large amounts of archaeal DNA in this sample. Agarose plugs prepared from this picoplankton sample were chosen for subsequent fosmid library preparation. Each 1 ml agarose plug from this site contained approximately 7.5×10^5 cells, therefore approximately 5.4×10^5 cells were present in the 72 ml slice used in the preparation of the partially digested DNA.

Vector arms are prepared from pFOS1 as described (Kim *et al.*, Stable propagation of cosmid sized human DNA inserts in an F factor based vector, *Nucl. Acids Res., 20:*10832-10835, 1992). Briefly, the plasmid is completely digested with AstII, dephosphorylated with HK phosphatase, and then digested with BamHI to generate two arms, each of which contains a *cos* site in the proper orientation for cloning and packaging ligated DNA between 35-45 kbp. The partially digested picoplankton DNA is ligated overnight to the pFOS1 arms in a 15 ml ligation reaction containing 25 ng each of vector and insert and 1U of T4 DNA ligase (Boehringer-Mannheim). The ligated DNA in four microliters of this reaction is *in vitro* packaged using the Gigapack XL packaging system (Stratagene), the fosmid particles transfected to *E. coli* strain DH10B (BRL), and the cells spread onto LB_{cm15} plates. The resultant fosmid clones are picked into 96-well microliter dishes containing LB_{cm15} supplemented with 7% glycerol. Recombinant fosmids, each containing 40 kb of picoplankton DNA insert, have yielded a library of 3,552 fosmid clones, containing approximately 1.4×10^8 base pairs of cloned DNA. All of the clones examined contained inserts ranging from 38 to 42 kbp. This library is stored frozen at -80°C for later analysis.

Example 4

Normalization of DNA from Environmental Samples

Prior to library generation, purified DNA from an environmental sample can be normalized. DNA is first fractionated according to the following protocol:

Sample composed of genomic DNA is purified on a cesium-chloride gradient. The cesium chloride (Rf = 1.3980) solution is filtered through a 0.2 mm filter and 15 ml is loaded into a 35 ml OptiSeal tube (Beckman). The DNA is added and thoroughly mixed. Ten micrograms of bis-benzimide (Sigma; Hoechst 33258) is added and mixed thoroughly. The tube is then filled with the filtered cesium chloride solution and spun in a VTi50 rotor in a Beckman L8-70 Ultracentrifuge at 33,000 rpm for 72 hours. Following centrifugation, a syringe pump and fractionator (Brandel Model 186) are used to drive the gradient through an ISCO UA-5 UV absorbance detector set to 280 nm. Peaks representing the DNA from the organisms present in an environmental sample are obtained.

Normalization is then accomplished as follows:

1. Double-stranded DNA sample is resuspended in hybridization buffer (0.12 M NaH_2PO_4, pH 6.8/0.82 M NaCl/1 mM EDTA/0.1% SDS).
2. Sample is overlaid with mineral oil and denatured by boiling for 10 minutes.
3. Sample is incubated at 68°C for 12-36 hours.
4. Double-stranded DNA is separated from single-stranded DNA according to standard protocols (Sambrook, 1989) on hydroxyapatite at 60°C.
5. The single-stranded DNA fraction is desalted and amplified by PCR.

The process is repeated for several more rounds (up to 5 or more).

Example 5

Hybridization Screening of Libraries Generated in Prokaryotes and Expression Screening in Metabolically Rich Hosts

Hybridization screening may be performed on fosmid clones from a library generated according to the protocol described in Example 3 above in any fosmid vector. For instance, the pMF3 vector is a fosmid based vector which can be used for efficient yet stable cloning in *E.coli* and which can be integrated and maintained stably in

Streptomyces coelicolor or *Streptomyces lividans*. A pMF3 library generated according to the above protocol is first transformed into *E.coli* DH10B cells. Chloramphenicol resistant transformants containing *tcm* or *oxy* are identified by screening the library by colony hybridization using sequences designed from previously published sequences of *oxy* and *tcm* genes. }(27, }28) Colony hybridization screening is described in detail in "Molecular Cloning", A Laboratory Manual, Sambrook, et al., (1989) 1.90-1.104. Colonies that test positive by hybridization can be purified and their fosmid clones analyzed by restriction digestion and PCR to confirm that they contain the complete biosynthetic pathway.

Alternatively, DNA from the abovementioned fosmid clones may be used in a amplification reaction designed to identify clones positive for an entire pathway. For example, the following sequences may be employed in an amplification reaction to amplify a pathway encoding the antibiotic gramicidin (gramicidin operon), which resides on a 34kbp DNA fragment potentially encoded on one fosmid clone:

Primers:

SEQ ID NO:1

5'CACACGGATCCGAGCTCATCGATAGGCATGTGTTTAACTTCTTGTCATC3'

SEQ ID NO:2

5'CTTATTGGATCCGAGCTCAATTGCTGAAGAGTTGAAGGAGAGCATCTTC C3'

Amplification reaction:

1 ml	fosmid/insert DNA
5 ml	each primer (50ng/ml)
1 ml	Boehringer Mannheim EXPAND Polymerase from their EXPAND kit
1 ml	dNTP's
5 ml	10X Buffer #3 from Boehringer Mannheim EXPAND kit

30 ml ddH_2O

PCR Reaction Program:

94°C 60 seconds
20 cycles of:
94°C 10 seconds
65°C 30 seconds
68°C 15minutes
one cycle of:
68°C 7minutes
Store at 4°C.

Fosmid DNA from clones that are shown to contain the oxytetracycline or tetracenomycin polyketide encoding DNA sequences are then used to transform *S. lividans* TK24 D*act* protoplasts from Example 6. Transformants are selected by overlaying regeneration plates with hygromycin (pMF5). Resistant transformants are screened for bioactivity by overlaying transformation plates with 2ml of nutrient soft agar containing cells of the test organisms *Escherichia coli* or *Bacillus subtilis* . *E. coli* is resistant to the thiostrepton concentration (50 mg/ml) to be used in the overlays of pMF3 clones but is sensitive to oxytetracylin at a concentration of 5 mg/ml }(29). The *B. subtilis* test strain is rendered resistant to thiostrepton prior to screening by transforming with a thiostrepton marker carried on pHT315 }(30). Bioactivity is demonstrated by inhibition of growth of the particular test strain around the *S. lividans* colonies. To confirm bioactivity, presumptive active clones are isolated and cultures extracted using a moderately polar solvent, methanol. Extractions are prepared by addition of methanol in a 1:1 ratio with the clone fermentation broth followed by overnight shaking at 4°C. Cell debris and media solids in the aqueous phase are then be separated by centrifugation. Recombinantly expressed compounds are recovered in the solvent phase and may be concentrated or diluted as necessary. Extracts of the clones

are aliquoted onto 0.25-inch filter disks, the solvent allowed to evaporate, and then placed on the surface of an overlay containing the assay organisms. Following incubation at appropriate temperatures, the diameter of the clearing zones is measured and recorded. Diode array HPLC, using authentic oxytetracyclin and tetracenomycin as standards, can be used to confirm expression of these antibiotics from the recombinant clones.

Rescue of chromosmally integrated pathways. Sequence analysis of chromosomally integrated pathways identified by screening can be performed for confirmation of the bioactive molecule. One approach which can be taken to rescue fosmid DNA from *S. lividans* clones exhibiting bioactivity against the test organisms is based on the observation that plasmid vectors containing IS*117*, such as pMF3, are present as circular intermediates at a frequency of 1 per 10-30 chromosomes. The presumptive positive clones can be grown in 25 ml broth cultures and plasmid DNA isolated by standard alkaline lysis procedures. Plasmid DNA preps are then used to transform *E. coli* and transformants are selected for Cm^r by plating onto LB containing chloramphenicol (15 mg/ml). Fosmid DNA from the *E. coli* Cm^r transformants is isolated and analyzed by restriction digestion analysis, PCR, and DNA sequencing.

Example 6

Host Strain Construction

The following example describes modifications that can be performed on the *Streptomyces lividans* strain to make it useful for screening bioactive clones originally identified in *E.coli* according to Example 5.

Streptomyces lividans is a strain is routinely used in the recombinant expression of heterologous antibiotic pathways because it recognizes a large number of promoters and appears to lack a restriction system. Although *Streptomyces lividans* does not normally produce the polyketide antibiotic actinorhodin, it contains the requisite gene sequences, and

several genes have been identified that activate its production in *S. lividans*. One strain of *S. lividans*, TK24, can be utilized as a host for screening for bioactive clones. This strain contains a mutation in the *rpsL* gene, encoding ribosomal protein S12, that confers resistance to streptomycin and activates the production of actinorhodin. In order to ensure that the bioactivity *of S. lividans* clones containing putative polyketide or other antibiotic genes is not due to the activation of the resident *act* gene cluster, these sequences should be removed from host strain by gene replacement. The outline for the gene replacement scheme is shown in Figure 8. Gene fragments internal to *act*VI and *act*VB, which define the boundaries of the *act* cluster are amplified by PCR. The primers used for the amplification have recognition sequences designed within them so that they are cloned in the proper orientation respective to each other and the *act* cluster. The *actVB* and *actVI* gene fragments are cloned into pLL25 so that they flank the spectinomycin encoding gene, generating pRBSV2. *S. lividans* TK24 protoplasts are transformed with pRBSV2 using established transformation protocols and transformants are selected for spectinomycin resistance. As shown in Figure 9, Spc^r transformants can arise as a result of several recombination events. Single recombination events within *act*VI or *act*VB (events 1 and 2) result in the insertion of the plasmid construct within the *act* cluster. A double crossover within *act*VI and *act*VB (recombination event 3) results in the replacement of the *act* cluster with the Spc^r encoding gene. While both types of recombinations can generate an Act$^-$ strain, the present example focuses on the construction of a strain containing the gene replacement. This is advantageous for two reasons: first, it generates a stable Act$^-$ strain that cannot revert to Act$^+$ by recombination between repeated sequences, and second, it decreases the amount of potential homology between cloned sequences and the chromosome, and decreases the likelihood of cloning partial pathways. Because the actinorhodin antibiotic is pigmented, one is able to distinguish the different classes of recombinants based on the pigment produced by the Spc^r transformants. Only Spc^r transformants that are generated by double recombination are non-pigmented. *S. lividans* TK24 clones that have the *act* cluster replaced by *spc* are confirmed by Southern hybridization and PCR analysis using standard techniques.

Example 7

Screening of Large Insert Library for Compounds of Interest

Large insert libraries generated according to Examples 1 and 3 can be screened for potentially clinically valuable compounds of interest using the following method(s):

Organic Extraction of Fosmid Library Clones (aqueous):

Add equal volume of Methyl-Ethyl-Ketone (MEK)(Sigma Chemical Co.) to each well of the microtiter plate from Example 3. Transfer MEK phase to new plates. Spin plates to dry down. Resuspend sample(s) in TN Buffer (50mM Tris-7, 10mM NaCl).

Protein Extraction of Streptomycine

1. Inoculate 25ml Trypticase Soy Broth (BBL Microbiology Systems) in 250 ml baffled erlenmeyer flasks with spores of Streptomyces lividans TK24. Incubate at 30°C at 225rpm for 48 hours.
2. Spin @ 4000 rpm in 50 ml conical to pellet cells (15 minutes).
3. Pour off supernatent and reserve.
4. Microscopically check pellet and supernatant.
5. Sonicate pellet
6. Pellet cell debris 4000rpm/15 minutes (reserve).
7. Pull off supernatant.
8. Dialyze against 80% saturated Ammonium Sulfate solution according to manufacturers instructions (Slide-A-Lyzer™ Dialysis from Pierce.
9. Spin prep at 2500 rpm for 15 minutes.
10. Spin prep again at 3500 rpm for another 15 minutes.
11. Pull of supernatant and reserve.
12. Add 1 ml TN buffer (50mM Tris pH 7; 100mM NaCl)

In 1.5ml screw caps, combine 50 l aqueous extract from fosmid clones with 50 l protein extract of Streptomycine (1:1 ratio) in assay wells.

Use different ratios of aqueous extract:protein extract (1:1 as indicated above, 3:1, etc.), as desired.

Incubate at 30°C for 4 hours.

Bioassay

1. Spot 20 ml of sample onto filter disk.
2. Lay filter disk on previously generated assay plate (growth plate containing appropriate media to grow organism of interest, with an overlay of ~ 1 OD 600 of cells of test organism solidified into soft agar). Grow cells overnight at the appropriate incubation temperature for the test organism to grow. Identify clearing zones for positive results (inhibition of growth).

Claims

What is claimed is:

1. A method for identifying a desired activity encoded by a genomic DNA population comprising:
   (a) obtaining a single-stranded genomic DNA population;
   (b) contacting the single-stranded DNA population of (a) with a DNA probe bound to a ligand under conditions and for sufficient time to allow hybridization and to produce a double-stranded complex of probe and members of the genomic DNA population which hybridize thereto;
   (c) contacting the double-stranded complex of (b) with a solid phase specific binding partner for said ligand so as to produce a solid phase complex;
   (d) separating the solid phase complex from the single-stranded DNA population of (b);
   (e) releasing from the probe the members of the genomic population which had bound to the solid phase bound probe;
   (f) forming double-stranded DNA from the members of the genomic population of (e);
   (g) introducing the double-stranded DNA of (f) into a suitable host cell to produce an expression library containing a plurality of clones containing the selected DNA; and
   (h) screening the expression library for the desired activity.

2. The method of claim 1, wherein the genomic DNA population is derived from uncultivated or cultivated microorganisms.

3. The method of claim 2, wherein the uncultivated or cultivated microorganisms are isolated from an environmental sample.

4. The method of claim 3, wherein the microorganisms isolated from an environmental sample are extremophiles.

5. The method of claim 4, wherein the extremophiles are selected from the group consisting of thermophiles, hyperthermophiles, psychrophiles, halophiles, acidophiles, barophiles and psychrotrophs.

6. The method of claim 1, wherein the genomic DNA, or fragments thereof, comprise one or more operons, or portions thereof.

7. The method of claim 6, wherein the operons, or portions thereof, encodes a complete or partial metabolic pathway.

8. The method of claim 7, wherein the operons or portions thereof encoding a complete or partial metabolic pathway encodes polyketide synthases.

9. The method of claim 1, wherein the expression library containing a plurality of clones is selected from the group consisting of phage, plasmids, phagemids, cosmids, phosmids, viral vectors and artificial chromosomes.

10. The method of claim 1, wherein the a suitable host cell is selected from the group consisting of a bacterium, fungus, plant cell, insect cell and animal cell.

11. The method of claim 1, wherein the DNA probe bound to a ligand is comprised of at least a portion of the coding region sequence of DNA for a known bioactivity.

12. The method of claim 1, wherein the ligand is selected from the group consisting of antigens or haptens, biotin or iminobiotin, sugars, enzymes, apoenzymes homopolymeric oligonucleotides and hormones.

13. The method of claim 1, wherein the binding partner for said ligand is selected from the group consisting of antibodies or specific binding fragments thereof, avidin or streptavidin, lectins, enzyme inhibitors, apoenzyme cofactors, homopolymeric oligonucleotides and hormone receptors.

14. The method of claim 1, wherein a solid phase is selected from the group consisting of a glass or polymeric surface, a packed column of polymeric beads or magnetic or paramagnetic particles.

15. The method of claim 1, further comprising producing an extract of the expression library.

16. The method of claim 15, further comprising combining the expression library extract with an enzyme extract from a metabolically rich host organism.

17. The method of claim 16, wherein the host organism is Streptomyces.

18. The method of claim 16, wherein the host organism is Bacillus.

19. A method for preselecting a desired DNA from a genomic DNA population comprising:

(a) obtaining a single-stranded genomic DNA population;

(b) contacting the single-stranded DNA population of (a) with a ligand-bound oligonucleotide probe that is complementary to a secretion signal sequence unique to a given class of proteins under conditions permissive of hybridization to form a double-stranded complex;

(c) contacting the double-stranded complex of (a) with a solid phase specific binding partner for said ligand so as to produce a solid phase complex;

(d) separating the solid phase complex from the single-stranded DNA population of (a);

(e) releasing the members of the genomic population which had bound to said solid phase bound probe;

(f) separating the solid phase bound probe from the members of the genomic population which had bound thereto;

(g) forming double-stranded DNA from the members of the genomic population of (e);

(h) introducing the double-stranded DNA of (g) into a suitable host cell to form an expression library containing a plurality of clones containing the selected DNA; and

(i) screening the expression library for the desired activity.

20. The method of claim 19, wherein the genomic DNA population is derived from uncultivated or cultivated microorganisms.

21. The method of claim 20, wherein the uncultivated or cultivated microorganisms are isolated from an environmental sample.

22. The method of claim 21, wherein the microorganisms isolated from an environmental sample are extremophiles.

23. The method of claim 22, wherein the extremophiles are selected from the group consisting of thermophiles, hyperthermophiles, psychrophiles, halophiles, acidophiles, barophiles and psychrotrophs.

24. The method of claim 19, wherein the genomic DNA, or fragments thereof, comprise one or more operons, or portions thereof.

25. The method of claim 24, wherein the operons, or portions thereof, encodes a complete or partial metabolic pathway.

26. The method of claim 25, wherein the operons or portions thereof encoding a complete or partial metabolic pathway encodes polyketide synthases.

27. The method of claim 19, wherein the expression library containing a plurality of clones is selected from the group consisting of phage, plasmids, phagemids, cosmids, phosmids, viral vectors and artificial chromosomes.

28. The method of claim 19, wherein the a suitable host cell is selected from the group consisting of a bacterium, fungus, plant cell, insect cell and animal cell.

29. The method of claim 19, wherein the DNA probe bound to a ligand is comprised of at least a portion of the coding region sequence of DNA for a known bioactivity.

30. The method of claim 19, wherein the ligand is selected from the group consisting of antigens or haptens, biotin or iminobiotin, sugars, enzymes, apoenzymes homopolymeric oligonucleotides and hormones.

31. The method of claim 19, wherein the binding partner for said ligand is selected from the group consisting of antibodies or specific binding fragments thereof, avidin or streptavidin, lectins, enzyme inhibitors, apoenzyme cofactors, homopolymeric oligonucleotides and hormone receptors.

32. The method of claim 19, wherein a solid phase is selected from the group consisting of a glass or polymeric surface, a packed column of polymeric beads or magnetic or paramagnetic particles.

33. The method of claim 19, further comprising producing an extract of the expression library.

34. The method of claim 33, further comprising combining the expression library extract with an enzyme extract from a metabolically rich host organism.

35. The method of claim 34, wherein the host organism is Streptomyces.

36. The method of claim 34, wherein the host organism is Bacillus.

37. A method for identifying a desired activity encoded by a nucleic acid population comprising:

a) generating one or more gene libraries derived from the nucleic acid population;

b) combining the extracts of the gene library or gene libraries generated in a) with target cell components obtained from metabolically rich cells; and

c) screening the combination of b) to identify the desired activity.

38. The method of claim 37, further comprising transforming host cells with recovered gene libraries derived from the nucleic acid population to produce an expression library of a plurality of clones.

39. The method of claim 37, wherein the target cell components are contained in a crude extract obtained from metabolically rich cells.

40. The method of claim 37, wherein the target cell components are purified proteins obtained from metabolically rich cells.

41. The method of claim 37, wherein the gene library extract and target cell components are co-encapsulated in a micro-environment.

42. The method of claim 41, wherein the micro-environment is a liposome, gel microdrop, bead, agarose, cell, ghost red blood cell or ghost macrophage.

43. The method of claim 42, wherein the liposomes are prepared from one or more phospholipids, glycolipids, steroids, alkyl phosphates or fatty acid esters.

44. The method of claim 43, wherein the phospholipids are selected from the group consisting of lecithin, sphingomyelin and dipalmitoyl.

45. The method of claim 44, wherein the steroids are selected from the group consisting of cholesterol, cholestanol and lanosterol.

46. The method of claim 37, wherein the activity encoded by a nucleic acid population is an enzyme or small molecule.

47. The method of claim 46, wherein the enzyme is selected from the group consisting of lipases, esterases, proteases, glycosidases, glycosyl transferases, phosphatases, kinases, mono- and dioxygenases, haloperoxidases, lignin peroxidases, diarylpropane peroxidases, epozide hydrolases, nitrile hydratases, nitrilases, transaminases, amidases, and acylases.

48. A method for identifying a desired activity encoded by a nucleic acid population obtained from a prokaryotic organism(s) comprising:

a) generating one or more gene libraries derived from the nucleic acid population;

b) combining the extracts of the gene library or gene libraries generated in a) with target cell components obtained from metabolically rich cells; and

c) screening the combination of b) to identify the desired activity.

49. The method of claim 48, further comprising transforming host cells with recovered gene libraries derived from the nucleic acid population to produce an expression library of a plurality of clones.

50. The method of claim 48, wherein the organisms are microorganisms.

51. The method of claim 50, wherein the microorganisms are uncultured microorganisms.

52. The method of claim 51, wherein the uncultured microorganisms are derived from an environmental sample.

53. The method of claim 51, wherein the uncultured microorganisms comprise a mixture of terrestrial microorganisms or marine microorganisms or airborne microorganisms, or a mixture of terrestrial microorganisms, marine microorganisms and airborne microorganisms.

54. The method of claim 51, wherein the uncultured microorganisms comprise extremophiles.

55. The method of claim 54, wherein the extremophiles are selected from the group consisting of thermophiles, hyperthermophiles, psychrophiles, barophiles, and psychrotrophs.

56. The method of claim 49, wherein the clones comprise a construct selected from the group consisting of phage, plasmids, phagemids, cosmids, fosmids, viral vectors, and artificial chromosomes.

57. The method of claim 48, further comprising screening the expression library for the specified enzyme activity.

58. The method of claim 48, wherein screening is by FACS analysis.

59. The method of claim 49, wherein the host cell is selected from the group consisting of a bacterium, fungus, plant cell, insect cell and animal cell.

60. The method of claim 48, wherein the gene library extract and target cell components are co-encapsulated in a micro-environment.

61. The method of claim 60, wherein the micro-environment is a liposome, gel microdrop, bead, agarose, cell, ghost red blood cell or ghost macrophage.

62. The method of claim 61, wherein the liposomes are prepared from one or more phospholipids, glycolipids, steroids, alkyl phosphates or fatty acid esters.

63. The method of claim 62, wherein the phospholipids are selected from the group consisting of lecithin, sphingomyelin and dipalmitoyl.

64. The method of claim 62, wherein the steroids are selected from the group consisting of cholesterol, cholestanol and lanosterol.

65. The method of claim 52, wherein the population of microorganisms is collected using a device comprising a solid support supporting a selectable microbial enrichment media.

66. The method of claim 65, wherein the selectable microbial enrichment media comprises a microbial attractant.

67. The method of claim 66, wherein the microbial attractant is selected from the group consisting of glucosamine, cellulose, pentanoic or other acids, xylan, lignin, chitin, alkanes, aromatics, chloroorganics, sulphonyls and heavy metals.

68. The method of claim 65, wherein the selectable microbial enrichment media comprises a growth inhibitor for eukaryotic organisms.

69. The method of claim 68, wherein a growth inhibitor specific for eukaryotic organisms is selected from the group consisting of nystatin, cycloheximide and pimaricin.

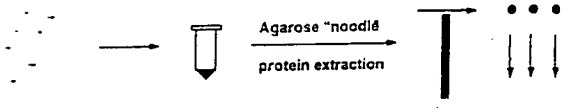
70. The method of claim 65, wherein the selectable microbial enrichment media comprises a growth inhibitor for prokaryotic organisms.

71. The method of claim 70, wherein a growth inhibitor specific for prokaryotic organisms is selected from the group consisting of polymyxin, penicillin and rifampin.

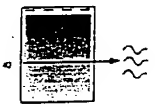
72. The method of claim 65, wherein the solid support is selected from the group consisting of glass beads, silica aerogels, agarose, Sepharose, Sephadex, nitrocellulose, polyethylene, dextran, nylon, natural and modified cellulose, polyacrylamide, polystyrene, polypropylene, and microporous polyvinylidene difluoride membrane.

Capturing Large Genome Fragments From the Environment

1. Concentrate bacteria, digest protein and preserve high MW (> 100 kbp) DNA



2. Partially digest DNA. Separte by PFGE. Size select for cloning.



3. Ligate to fosmid arms λ package and transfect to *E. coli* Array library in microtiter plates.

Figure 1. Scheme to capture, clone and archive large genome fragments from uncultivated microbes from natural environments. The cloning vectors used in this process can archive from 40 kbp (fosmids) to greater than 100 kbp (BACs).

Saccharopo 352 (SEQ ID NO:3)

GCCGCCGACACCCCGATCACGCCGATCGTGGTGTCCTGCTTCGACGCCA

TCAAGGCGACC

coelicolor 343 (SEQ ID NO:4)

GCCGCCGACACCCCGATCACCCCGATCGTCGTCGCCTGCTTCGACGCGA

TCCGCGCCAC

Gvenzuelae 337 (SEQ ID NO:5)

TCCTCGGACGCCCCGATCTCCCCGATCACGATGGCCTGCTTCGACGCCA

TCAAGGCGACC

fraidiae 352 (SEQ ID NO:6)

GCGGCCGACGCCCCGATCTCGCCCATCACCGTGGCCTGCTTCGATGCGA

TCAAGGCGACC

glaucescen 343 (SEQ ID NO:7)

GCCACCGACGCGCCGATCTCCCCCATCACCGTGGCCTGCTTCGACGCCA

TCAAGGCGAC

Ggriseus 352 (SEQ ID NO:8)

GCGGTGGACGCGCCGATCACCCCGCTCACGATGGCGGCCTTCGACGCGA

TCCGCGCCACC

E.coli 340 (SEQ ID NO:9)

GGCGCAGAGAAAGCCAGTACGCCGCTGGGCGTTGGTGGTTTTGGCGCG

GCACGTGCATTA

Figure 2

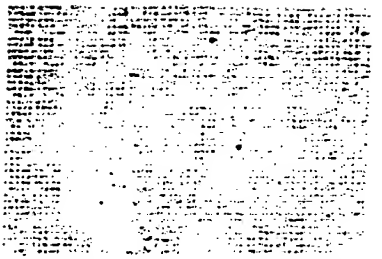


Figure 3. Example of a high density filter array of environmental fosmid clones probed with a labeled oligonucleotide probe. The 2400 arrayed clones contain approximately 96 million base pairs of DNA cloned from a naturally occurring microbial community.

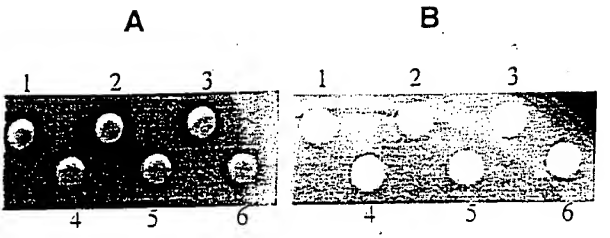


Figure 4. Results of mixed extract experiment measuring conferral of bioactivity on recombinant backbones heterologously expressed in *E. coli*.. A. Organic extracts from 3 oxytetracylin clones (1-3) and 3 gramicidin clones (4-6) were incubated with a protein extract from *Streptomyces lividans* strain TK24. After incubation the mixture was reextracted with methyl ethyl ketone, spotted on to filter disks, allowed to dry, then placed on a lawn of an *E. coli* test strain. Distinct zones of clearing can be seen around disks 2,3 and 5. Extracts from 2 and 3 were subsequently seperated by thin layer chromatography which showed UV fluorescent spots with similar Rf and appearance to authentic oxytetracylin. **B**. Filters corresponding to those in A but without incubation with protein extract from *Streptomyces*. The *Streptomyces* extract alone also showed no bioactivity.

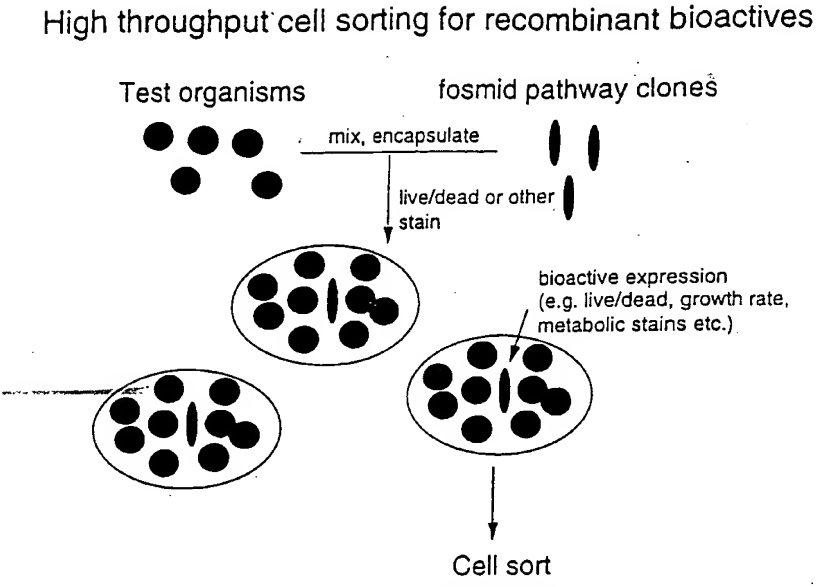


Figure . Strategy for FACS screening for recombinant bioactive molecules in *Streptomyces venezuelae*.

FIGURE 5

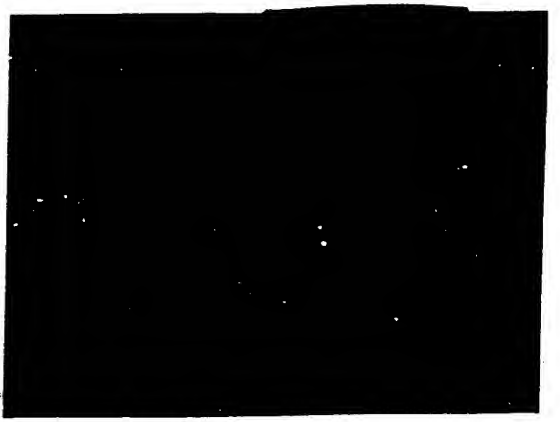


Figure . Micrograph of pMF4 oxytetracyclin clone expressed in *S. lividans* strain TK24. The red fluorescence near the end of the mycelia suggests that recombinant expression of oxytetracyclin may be induced at the onset sporulation as is the activity of the endogenous actinorhodin pathway.

FIGURE 6

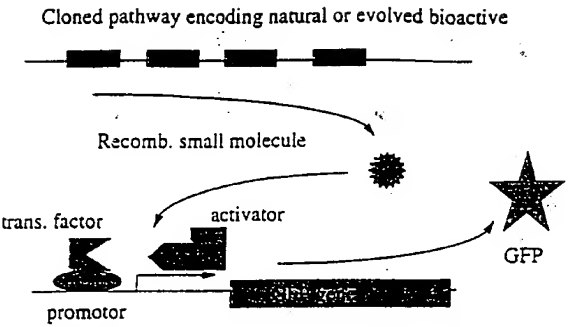


Figure . Approach to screen for small molecules that enhance or inhibit transcription factor initiation. Both the small-molecule pathway and the GFP reporter construct are co-expressed. Clones altered in GFP expression can then be sorted by FACS and the pathway clone isolated for characterization.

FIGURE 7

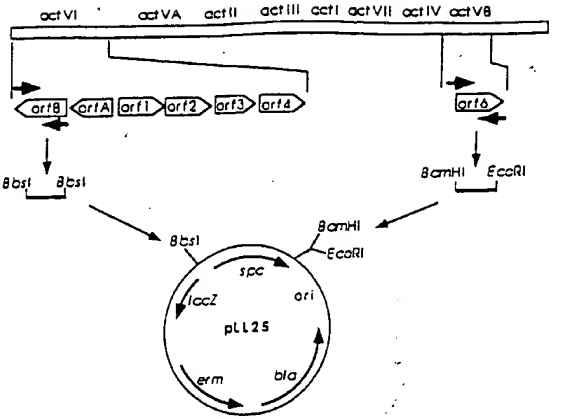


Figure . Gene replacement vector pLL25 designed to inactivate the actinorhodin pathway in *Streptomyces lividans* strain TK24.

FIGURE 8

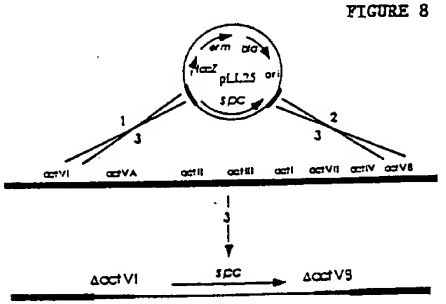


Figure . Possible recombination events and predicted phenotypes from replacement of the actinorhodin gene cluster in *S. lividans* by the spectinomycin gene resident on pLL25.

Recombination Event	Expected Phenotype	
1	Spc^r	Blue
2	Spc^r	Brown
3	Spc^r	Unpigmented

FIGURE 9

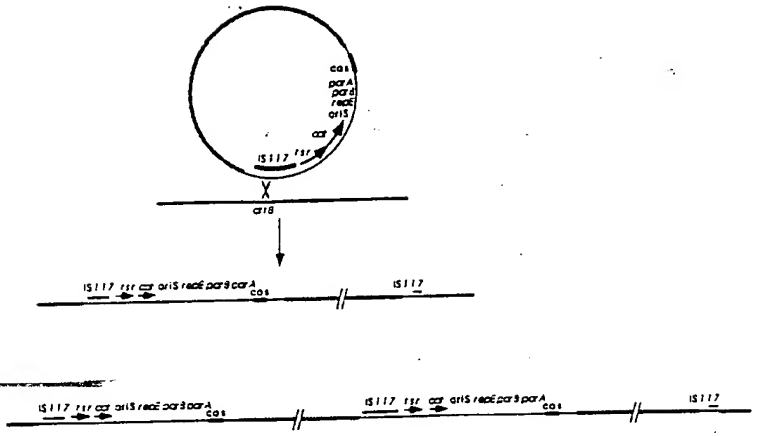


Figure . Tandem duplication of a pMF3 clone into the S. lividans chromosome. Duplicated clones will contain *cos* sites at the appropriate spacing for lambda packaging.

FIGURE 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/17779

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.2, 172.3; 536/23.1, 24.3, 24.31, 24.33, 25.4; 436/501

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 5,783,431 A (PETERSON et al) 21 July 1998, see entire document.	1-72
Y	Biomagnetic techniques in molecular biology. Dynal Technical Handbook, second edition. 1995. pages 78-89, see entire document.	1-72
Y	NAKAMURA et al. The murine lymphotoxin-B receptor cDNA: isolation by the signal sequence trap and chromosomal mapping. Genomics. 1995. Vol. 30, pages 312-319, see entire document.	1-72

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 SEPTEMBER 1998	Date of mailing of the international search report 30 OCT 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEFFREY FREDMAN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*